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㉗ **Nucleic acid encoding the alpha or beta chains of inhibin and method for synthesizing polypeptides using such nucleic acid.**

㉘ **DNA encoding the prepro inhibin α and β chains has been isolated. This DNA is ligated into expression vectors and used to transform host cells for the preparation of inhibin or activin. Also provided are prohormone domains and other inhibins α or β chain derivatives having therapeutic or diagnostic interest. The compositions provided herein are useful in the manipulation of fertility in animals.**

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NUCLEIC ACID ENCODING THE α OR β CHAINS OF INHIBIN AND METHOD FOR SYNTHESIZING POLYPEPTIDES USING SUCH NUCLEIC ACID

BACKGROUND

This invention relates to methods for making proteins in recombinant cell culture which contain the α or β chains of inhibin. In particular, it relates to methods for obtaining and using DNA which encodes inhibin, and for making inhibin variants that depart from the amino acid sequence of natural animal or human inhibins and the naturally-occurring alleles thereof.

Inhibin is a protein produced in the gonad which acts specifically at the pituitary level to inhibit the secretion of follicle-stimulating hormone - (FSH). The existence of inhibin was first postulated by McCullagh in 1932 ("Science" 76: 19-20). Such preferential regulation of the gonadotropin secretion has generated a great deal of interest and has prompted many laboratories in the past fifty years to attempt to isolate and characterize this substance from extracts of testis, spermatozoa, rete testis fluid, seminal plasma and ovarian follicular fluid, using various bioassays. Although many reports have appeared in the literature claiming the purification of inhibin-like material with molecular weights ranging from 5,000 to 100,000 daltons, subsequent studies have shown that these substances were not homogenous, did not have the high specific activity expected of true inhibin and/or failed to exhibit the molecular characteristics of inhibin as described herein (de Jong, Inhibin-Factor Artifact, "Molecular & Cellular Endocrin." 13: 1-10 - (1979); Sheth *et al.*, 1984, "F.E.B.S." 185(1) 11-15; Seidah *et al.*, 1984, "F.E.B.S." 175(2):349-355; Lijja *et al.*, March 1985, "F.E.B.S." 182(1):181-184; Li *et al.*, June 1985, "Proc. Nat. Acad. Sci. USA" 82:4041-4044; Seidah *et al.*, "F.E.B.S." 167(1):98-102; and Beksac *et al.*, 1984, "Intern. J. Andrology" 7:389-397).

A polypeptide having inhibin activity was purified from bovine or ovine follicular fluid (PCT 86/00078, published January 3, 1986). This protein was reported to have a molecular weight of 56,000 \pm 1,000 on SDS-PAGE and was dissociable into two subunits having apparent molecular weights of 44,000 \pm 3,000 and 14,000 \pm 2,000. Amino terminal sequences for each subunit were described.

Two proteins both having a molecular weight of about 32,000 daltons and having inhibin activity have been successfully isolated from porcine follicular fluid. Purification of porcine inhibin to substantial homogeneity, i.e., about 90% by weight of total protein in the fraction, was achieved through a

combination of protein separation procedures including heparin-Sepharose affinity chromatography, gel filtration and reverse-phase, high-performance liquid chromatography (RP-HPLC).

These proteins were isolated to substantial homogeneity from material obtained from swine and are referred to as Protein A and Protein B. Each protein has a molecular weight of about 32,000 daltons (32K) and is composed of two polypeptide chains having molecular weights of 18,000 and 14,000 daltons, respectively, the chains being linked together in the hormonally-active protein by disulfide bonding. The amino-terminal amino acid residue sequence of the 18,000 daltons (18K) or alpha chain of both proteins was determined to be Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val. The amino-terminal amino acid residue sequence of the 14,000 dalton (14K) or beta chain of Protein A was determined to be Gly-Leu-Glu-X-Asp-Gly-Lys-Val-Asn-Ile-X-X-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala and of Protein B was determined to be Gly-Leu-Glu-X-Asp-Gly-Arg-Thr-Asn-Leu-X-X-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu. Proteins A and B have been completely characterized. Each 32K protein exhibits inhibin activity in that it specifically inhibits the basal secretion of FSH but does not inhibit secretion of luteinizing hormone (LH). The individual chains were not hormonally-active.

After the filing of the parent application hereto, inhibin B-chain dimers were shown to exist in follicular fluid as naturally-occurring substances, termed activin, which are capable of stimulating FSH release by rat anterior pituitary cells (Vale *et al.*, 1986, "Nature" 321:776-779 and Ling *et al.*, 1986, "Nature" 321:779-782).

The amino acid sequence of the α and β chains of inhibin from humans remained unknown until the invention herein. The large quantities of human follicular fluid required to parallel the studies conducted with animal inhibins are not readily available, nor is there any assurance that human and animal inhibins would be sufficiently similar that purification using a parallel procedure would be effective. Accordingly, methods are needed for determining the characteristics and amino acid sequence for human inhibin.

Also needed are economical methods for making the α and β chains of inhibin in large quantities, preferably entirely and completely free of proteins from the species homologous to the inhibin in question, which inhibin preferably also is biologically active.

These and other objects will be apparent from consideration of the invention as a whole.

SUMMARY

Nucleic acid now has been isolated and cloned in replicable vectors which encodes the mature porcine and human α and β chains of inhibin and their precursor prepro and pro forms. Sequencing of inhibin-encoding cDNA has led to the identification of prodomain regions located N-terminal to the mature inhibin chains that represent coordinately expressed biologically active polypeptides. The prodomain regions or prodomain immunogens are useful in monitoring preproinhibin processing in transformant cell culture or in experiments directed at modulating the clinical condition or reproductive physiology of animals. Thus α or β chain nucleic acid is used to prepare prodomain sequences from the precursor forms of the inhibin chains, to transform host cells for the recombinant expression of mature inhibin α and/or β chains, and in diagnostic assays. In particular, regions from inhibins α and/or β chains are expressed in recombinant cell culture by a method comprising ligating the nucleic acid encoding the region into a replicable vector under the control of a promoter, transforming a host cell with the vector, culturing the host cell and recovering the prodomain, activin or inhibin from the cultured cell. Inhibin, activin and prod domains produced by the method of this invention are entirely free of homologous source proteins and can be produced in biologically active form.

The nucleic acids identified herein encode the α , β_A and β_B chains of porcine or human inhibin. Recombinant cells are transformed to express $\alpha\beta_A$ or $\alpha\beta_B$ inhibins, or to express β -chain heterodimers or homodimers (which are collectively referred to in the literature as activin). β -chain dimers as products of recombinant cells expression are free of homologous proteins with which they ordinarily are associated in nature.

Inhibin or activin and their nontoxic salts, combined with a pharmaceutically acceptable carrier to form a pharmaceutical composition, are administered to mammals, including humans, for control of fertility. Administration of inhibin decreases fertility in female mammals and decreases spermatogenesis in male mammals, and administration of a sufficient amount induces infertility. Inhibin is also useful in tests to diagnose infertility. Activin has been shown in the literature to be capable of stimulating FSH release from pituitary cells and accordingly is useful as a fertility inducing therapeutic.

The method of this invention also facilitates the convenient preparation of inhibin, activin and prodomain variants having primary amino acid sequences and/or glycosylation differing from the native analogues, in particular fusions of immunogenic peptides with inhibin, activin or prodomain sequences.

Brief Description of the Drawings

Fig. 1A is a schematic representation of the porcine α -chain mRNA. Overlapping cDNA clones used in the sequence determination are shown above the diagram of the mRNA structure. Black boxes on the 3' ends of λ clones indicate that these clones were obtained by specific priming. Untranslated sequences are represented by a line, coding sequences are boxed. The unfilled portion represents the coding region for the signal peptide and pro-sequences, and the cross-hatched areas indicates the 134 amino acid α -chain. The scale is in nucleotides from the 5' end of the longest cDNA clone.

Fig. 1B shows the nucleotide and predicted amino acid sequence of the porcine α -chain precursor. Nucleotides are numbered at the left and amino acids are numbered throughout. The amino acid sequence underlined was used to design a long synthetic DNA probe. The 364 amino acid precursor includes a hydrophobic signal sequence, a pro-region, and the mature α -chain (amino acids 231-364). The proteolytic processing site Arg-Arg (black bar) immediately precedes the NH₂-terminus of the mature alpha chain. Several other putative dibasic processing sites present in the pro-region are indicated by open bars. The two potential N-linked glycosylation sites are shown by the cross-hatched bars. The AATAAA box close to the 3' end of the mRNA is underlined.

Fig. 2A is a schematic representation of the porcine β_A and β_B subunit mRNAs with coding sequences boxed. The β_A and β_B subunits (dashed) are encoded towards the 3' end of the coding sequences. The 3' and 5' untranslated regions are shown as a line. The length of the 5' and 3' untranslated region of the β_B subunit mRNA is inferred from the size of the mRNA (Fig. 3) and its obvious similarity to the β_A mRNA. Tentative regions of the cDNAs are shown as dashed in the diagram. The relative positions of the overlapping oligo-dT primed cDNA clones and the randomly primed clones (λ PIN β_A 5s, λ PIN β_B 1s, and λ PIN β_B 2s) are indicated. The scale is in nucleotides from the 5' end of the 4.5 kb mRNA.

Fig. 2B is the nucleotide sequence and deduced amino acid sequence of the porcine inhibin β -subunit precursors. The β_B sequence is aligned

with the β_A sequence for maximum homology. The NH₂-terminal of the β -subunit precursors are indicated by bracket and arrows. Cysteine residues are shaded, possible processing sites are indicated by open bars, and a potential glycosylation site is shown by the cross-hatched box. A very GC-rich region present 3' to the termination codon intron sequences is underlined and overlined in both sequences. Amino acid sequences used to design oligonucleotide probes are underlined, as is the AATAAA polyadenylation signal. There was one nucleotide difference between λ PIN- β_A 8 and other clones covering this area. A G-to-A change causes a change of amino acid 278 from a glycine to a serine. The proteolytic processing site Arg Arg Arg Arg Arg (black bar) immediately precedes the NH₂ terminus of the mature β_A subunit, with the prosequences located upstream. The amino acids for the β_A subunit only are numbered.

Fig. 3 is a Northern blot analysis of porcine ovarian mRNA with α , β_A and β_B subunit cDNA hybridization probes. Lanes a, b, c, d, and f are polyA⁺ mRNA and e and g are total RNA. The position of the 28S and 18S ribosomal RNAs are shown. Lanes a, d, and e were hybridized with an α -subunit cDNA probe; lanes d, e and g with a β_A subunit specific probe, and lane c with a β_B subunit specific probe. The α -subunit mRNA is approximately 1.5 kb, the β_A subunit mRNAs are approximately 4.5 kb. The hybridizations shown in lanes a, b, and c were performed with probes of approximately equal length and specific activity in order to judge relative mRNA levels.

Fig. 4A is a comparison of the human β -TGF amino acid sequence and porcine inhibin β_A and β_B amino acid sequences. The sequences were aligned around the cysteine residues. Identical residues are boxed, while conservative changes are designated by an asterisk.

Fig. 4B compares the α -subunit sequence with the β_A -inhibin sequence.

Fig. 5 depicts the construction of a representative recombinant expression plasmid for porcine inhibin.

Fig. 6 shows the nucleotide sequence and deduced amino acid sequence of the human α -inhibin cDNA. The 335 amino acid pro- α inhibin sequence is numbered from the hypothesized signal cleavage site. Sixteen amino acids of the signal sequence are numbered -1 through -16. Homology with the porcine sequence predicts a further 12 amino acid residues in the signal sequence. In this and other figures, putative dibasic processing sites are shown by the open bars, glycosylation sites indicated by cross-hatched bars, and amino terminal mature chain processing sites are depicted as black bars. The poly(A) additional signal sequence is underlined. Cysteine residues are shaded.

Fig. 7 is a comparison of the human and porcine α -inhibin protein sequences. Spaces are introduced to maximize the homology; positions of non-identity are indicated by stars. Numbering is as for the porcine sequence, which is one amino acid shorter than the human.

Fig. 8 shows that the nucleotide and deduced amino acid sequence of the human β_A inhibin signal sequence (residues -28 through -1) is 28 amino acids with the precursor being 378 amino acids in length. The basic processing site is indicated by a black bar, and a potential glycosylation site in the precursor is indicated by a cross-hatched bar above the sequence. Cysteine residues are shaded.

Fig. 9 illustrates the nucleotide and deduced amino acid sequence of human β_B inhibin cDNA. The sequence commences at a cysteine residue - (position 7), which lines up with the cysteine present at residue 7 in the β_A sequence (see Fig. 8). The processing site for the mature β_B inhibin is shown as a black bar and a potential glycosylation site as a cross-hatched bar. Cysteine residues are shaded.

DETAILED DESCRIPTION

The polypeptides of this invention are the α and β chains of inhibin, as well as their multimer forms (activin and inhibin), their prepro forms and their prodomains, together with glycosylation and/or amino acid sequence variants of each chain or form thereof. Inhibin (including alleles) from human or animal sources inhibits the basal release of FSH but not of LH from anterior pituitary cells while activin does the opposite (hereinafter referred to as "hormonally active" activin or inhibin).

Generally, amino acid sequence variants will be substantially homologous with the relevant portion of the porcine or human α or β chain sequences set forth in Figs. 1B, 2B, 6, 8 and 9. Substantially homologous means that greater than about 70% of the primary amino acid sequence of the candidate polypeptide corresponds to the sequence of the porcine or human chain when aligned in order to maximize the number of amino acid residue matches between the two proteins. Alignment to maximize matches of residue includes shifting the amino and/or carboxyl terminus, introducing gaps as required and/or deleting residues present as inserts in the candidate. For example, see Figs. 2B and 7 where the β_A and β_B subunits or human and porcine α -inhibin sequences are aligned for maximum homology. Typically, amino acid sequences variants will be greater than about 90% homologous with the corresponding native sequences shown in Figs. 1B, 2B, 6, 8 and 9.

Variants that are not hormonally-active fall within the scope of this invention, and include polypeptides that may or may not be substantially homologous with either a mature inhibin chain or prodomain sequence, but which are 1) immunologically cross-reactive with antibodies raised against the native counterpart or 2) capable of competing with such native counterpart polypeptides for cell surface receptor binding. Hormonally inactive variants are produced by the recombinant or organic synthetic preparation of fragments, in particular the isolated β chains of inhibin, or by introducing amino acid sequence variations so that the molecules no longer demonstrate hormonal activity as defined above.

Immunological or receptor cross-reactivity means that the candidate polypeptide is capable of competitively inhibiting the binding of the hormonally-active analogue to polyclonal antisera raised against the hormonally-active analogue. Such antisera are prepared in conventional fashion by injecting goats or rabbits S.C. with the hormonally-active analogue or derivative in complete Freund's adjuvant, followed by booster intraperitoneal or S.C. injections in incomplete Freund's.

Variants that are not hormonally active but which are capable of cross-reacting with antisera to hormonally-active inhibin, activin, or prod domains are useful (a) as reagents in diagnostic assays for the native analogues or their antibodies, (b) when insolubilized in accord with known methods, as an agent for purifying anti-native analogue antibodies from antisera, and (c) as an immunogen for raising antibodies to hormonally-active analogues.

This invention includes the pro and/or prepro sequences of the inhibin α or β chain precursors, or their immunologically or biologically active fragments, substantially free of the corresponding mature inhibin chains. These sequences for porcine and human inhibin are shown in Figs. 1B, 2B, 6, 8 and 9. The prepro sequence for the porcine α subunit precursor is the polypeptide comprised by residues 1 to about 230, while the β_A subunit pro sequence is comprised by residues 1 to about 308. These sequences shall be referred to herein as encompassing prodomain sequences.

The α and β subunit prodomain sequences are comprised of several domains bounded by proteolysis sites, any one of which is synthesized herein separately or in combination with other domains. The principal porcine β_A domains fall within residues 1 to about 70 (domain I), about 70 to about 110 (domain II), about 110 to about 180 (domain III), about 180 to about 260 (domain IV), and about 270 to about 309 (domain V). In particular, the porcine β_A domains are GHSAAPDCPSCALATLPK-DVPNSQPEMVEAV, HILNMLHLKRPDVTQVP-

KAALLNAI, LHVGVGVGENGYVELEDDIG, AEMNEL-MEQTSEITFAEAGRARKTLRFEISKEGSDLSVVER-AEIWLKVPKANRTRTKV, SIRLFQQQ, PQGSADAGEEAEDVGFPEEKSEVLISEKVDA, STWHIFPVSSSIQRLLDQGKSALDIRTACEQCHET-GASLVLLG, and GHSAAPDCPSCALATLPKDVPN-SQPEMVEAVKKHILNMLHLKRPDVTQVPKAALL-NAI. The porcine β_B domains comprise RAAHILL-HAVRVSGWLNL as well as homologous β domains having the same sequences. The porcine α domains comprise GPELDRELVLAKVRALFDALGP-PAVTGEGGDPGV and GSEPEEDVSQAILFPAT-GARCGAEPAAAGELAREAEGLFTYVGRPSQHTHS-RQVTSQQLWFHTGL DRQGMAAANSSGPLL-DL-LALSSRGPVAVPMSLGQAPPFWAVLHLAASALPL-LTHPVLVLLRCPLC SCSARPEATPFLVAHTRARP-PSGGERA. A typical combination domain polypeptide would be β_A domain II linked at its C-terminus to the NH₂-terminus of β_A domain III. In addition, these domains are fused together by the proteolysis sites found in the sequences shown in Figs. 1B or 2B, by 1 to 4 residue polypeptides that are resistant to hydrolysis (for example, glutamyl or histidyl residues), or are directly fused, whereby, in all three instances, combination domain polypeptides are produced.

Principal human α chain prod domains are approximately residues 30-199 and 1 to 29, human β_A prod domains are approximately residues 1-30, 32-40, 43-59, 62-90, 83-185 and 186-230 while human β_B prod domains are approximately residues 1-13, 15-30, 32-59, 62-145, 148-195 and 198-241 - (referring to the numbering system adopted in Figs. 6, 8 and 9, respectively). Combination prodomain polypeptides are within the scope hereof, for example, the β_A prodomain at about 43-80, and β_B prod domains at about 1-30 and about 32-145. The preferred human α , β_A and β_B chain prod domains are about residues 1-29, about 43-80 and about 1-30, respectively.

The intact isolated prepro or prodomain β_A , β_B or α sequences are best synthesized in recombinant cell culture. The individual subcomponent domains are synthesized by routine methods of organic chemistry or by recombinant cell culture. They then are labelled with a radioisotope or other detectable group such as an enzyme or fluorophore in accord with known methods and used in standard competitive immunoassays to detect the levels of prepro or pro forms of inhibin, including individual domains, in transformants with DNA encoding such forms or their precursors. This assay is useful in determining whether proteolytic hydrolysis of proinhibin is occurring in the host transformants or their culture media. The assay also is useful in determining whether a rate limiting step in recombinant synthesis is translation of mRNA into the prepro forms or processing of the

prepro forms into mature inhibin. For example, high levels of prepro or pro inhibin in cell lysates, but relatively low levels of secreted mature inhibin, would suggest that the host cell is adequately transcribing and translating the inhibin DNA, but is not processing the precursors at an adequate rate. Thus, in this case one would select an alternate host cell rather than concentrating on improving the transcription or translation efficiency of the transforming plasmid, e.g., by selecting an alternate promoter. The prodomain sequences also are believed to be involved in coordinate modulation of animal physiology in reproductive cycles and fertility.

Amino acid sequence variants are any one of 1) hormonally-active, 2) cross reactive with antibodies raised against mature inhibin or prodomain α or β chain sequences, or 3) cross-reactive with inhibin/activin cell surface receptors, but are characterized by a primary amino acid sequence that departs from the sequence of natural inhibins or prodomain sequences. These derivatives ordinarily are prepared by introducing insertions, deletions or substitutions of nucleotides into the DNA encoding the target DNA to be modified in order to encode the variant, and thereafter expressing the DNA in recombinant cell culture. Polypeptides having up to about 100-150 residues also are conveniently prepared by *in vitro* synthesis. Such variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation. The variants may exhibit the same qualitative biological activity as the naturally-occurring analogue or may act antagonistically towards such analogues.

While the site for introducing a sequence variation is predetermined, it is unnecessary that the mutation per se be predetermined. For example, in order to optimize the performance of mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed inhibin mutants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence is well known, for example M13 primer mutagenesis.

Mutagenesis is conducted by making amino acid insertions, usually on the order of about from 1 to 10 amino acid residues, or deletions of about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any subcombination may be combined to arrive at a final construct. Insertions include amino or carboxyl-terminal fusions, e.g. a hydrophobic extension added to the carboxyl terminus. Preferably, however, only substitution mutagenesis is conducted. Obviously, the muta-

tions in the encoding DNA must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Not all mutations in the DNA which encode the polypeptides herein will be expressed in the final secreted product. For example, a major class of DNA substitution mutations are those in which a different secretory leader or signal has been substituted for the native porcine or human α or β chain secretory leader, either by deletions within the leader sequence or by substitutions, wherein most or all of the native leader is exchanged for a leader more likely to be recognized by the intended host. For example, in constructing a procaryotic expression vector the porcine or human α or β chain secretory leader is deleted in favor of the bacterial alkaline phosphatase or heat stable enterotoxin II leaders, and for yeast the leader is substituted in favor of the yeast invertase, alpha factor or acid phosphatase leaders. However, the porcine and human secretory leaders are recognized by many heterologous higher eukaryotic cells. When the secretory leader is "recognized" by the host, the host signal peptidase is capable of cleaving a fusion of the leader polypeptide fused at its C-terminus to the mature inhibin or prodomain such that mature inhibin or prodomain polypeptide is secreted.

Another major class of DNA mutants that are not expressed in final form as amino acid sequence variations are nucleotide substitutions made in the DNA to enhance expression, primarily to avoid 5' stem and loop structures in the transcribed mRNA (see de Boer *et al.*, EP 75,444A) or to provide codons that are more readily transcribed by the selected host, e.g. the well-known preference codons for *E. coli* or yeast expression. These substitutions may or may not encode substituted amino acid residues, but preferably do not.

Insertional and deletional amino acid sequence variants are proteins in which one or more amino acid residues are introduced into or removed from a predetermined site in the target inhibin, activin, prodomain or proform of inhibin or activin. Most commonly, insertional variants are fusions of heterologous proteins or polypeptides to the amino or carboxyl terminus of the α or β chains, the prodomains or other inhibin derivatives. Immunogenic derivatives are made by fusing an immunogenic polypeptide to the target sequence, e.g. a prodomain polypeptide, by synthesis *in vitro* or by recombinant cell culture transformed with DNA encoding the fusion. Such immunogenic polypeptides preferably are bacterial polypeptides such as trpLE, beta-galactosidase and the like, together with their immunogenic fragments. Other insertions entail inserting heterologous eukaryotic (e.g. the herpes virus gD signal) or microbial secretion sig-

nal or protease processing sequences upstream from the NH₂-terminus of the protein to be secreted. Deletions of cysteine or other labile residues also may be desirable, for example in increasing the oxidative stability of the α or β chain. Deletion derivatives will produce α or β chain fragments. Such fragments, when biologically or immunologically active, are within the scope herein. For instance, a fragment comprising β_B or β_A residues about from 11 to 45 (numbered from mature Gly₁) is to be included within the scope herein.

Immunogenic conjugates of prodomain polypeptides, inhibin and activin are readily synthesized in recombinant cell culture as fusions with immunogenic polypeptides, e.g. beta-lactamase or viral antigens such as herpes gD protein, or by preparation of the polypeptides in unfused form (by recombinant or *in vitro* synthetic methods) followed by covalent cross-linking to an immunogenic polypeptide such as keyhole limpet hemocyanin or STI using a divalent cross-linking agent. The immunogenic polypeptides are formulated with a vaccine adjuvant, e.g. alum or Freund's. Methods for preparing proteins in adjuvants and for cross-linking are well-known per se and would be employed by one skilled in the art, as are methods for vaccinating animals. The immunogenic conjugates are useful in preparing antibodies to the prodomain region for use in monitoring inhibin manufacture or for *in vivo* vaccination with the objective of raising antibodies capable of modulating animal physiology in reproductive cycles and fertility. Typically, the prodomain or its immunogen is administered in varied doses to fertile laboratory animals or swine and the reproductive cycles and fertility of the animals monitored, together with assays of serum levels of anti-immunogen or prodomain by routine competitive or sandwich immunoassay.

Substitution derivatives are produced by mutating the DNA in a target codon, so that thereafter a different amino acid is encoded by the codon, with no concomitant change in the number of residues present in the molecule expressed from the mutated DNA. Substitutions or deletions are useful for example in increasing the stability of the proteins herein by eliminating proteolysis sites, wherein residues are substituted within or adjacent to the sites or are deleted from the sites, or by introducing additional disulfide bonds through the substitution of cysteine for other residues. Substitutions are useful for facilitating the synthesis or recovery of mature or prodomain α or β chains. For example, methionine residues within the mature inhibin sequences are substituted or deleted, prepro sequences deleted, methionine inserted at the -1 site immediately NH₂ terminal to the mature NH₂ terminal residue and another sequence inserted N-termi-

nal to the exogenous methionine. The inhibin derivative in this case is expressed as a fusion having an intermediate methionyl residue, which in turn is cleaved at this residue by cyanogen bromide in accordance with known practice. The mature inhibin derivative released from the fusion is recovered.

Exemplary porcine inhibin derivatives are - [Asn₂₈₈->Gln]Inh α (to remove the putative glycosylation site), [Cys₂₂₈ or Cys₂₃₇-> Δ]Inh α , [Cys₂₂₈ or Cys₂₃₇-> Δ]Inh α , [Lys₂₂₈ or Lys₂₃₇-> Δ]Inh β_A or - [Lys₂₂₈->His or Ser]Inh β_A (to inactivate a potential proteolysis site), [Lys₂₁₄->Arg; Val₂₁₄->Thr] Inh β_A (to create a β_A/β_B hybrid), [Cys₂₂₈ or Cys₂₃₇-> Δ]Inh β_A , [Lys₁₁₁->Gln]Inh β_A , [Arg₂₁₈->Lys; Val₂₁₄->Thr]Inh β_B - (to create a β_B/β_A hybrid), [Cys₂₁₈ or Cys₂₂₈-> Δ]Inh β_B [Pro₂₁₈Gly₂₂₈->Pro Phe Gly]Inh β_B , and [Arg₂₂₈->Gln]Inh β_B , wherein Inh is an abbreviation for inhibin and the residue members for Inh β_B are those used for the corresponding Inh β_A residue (see Fig. 2B).

The Inh β_A amino acid positions which are principal candidates for mutational substitution or deletion (or adjacent to which residues may be inserted) include residues 293-297, 364-376 and 387-398 (Fig. 8). Preferably, the proline, cysteine and glycine residues within these sequences are not modified. Candidates having greater potency than inhibin or activin, or which serve as inhibin or activin antagonists, are identified by a screening assay wherein the candidate is diluted into solutions containing constant amounts of inhibin or activin and the compositions assayed in the rat pituitary cell assay. Candidates which neither antagonize or agonize inhibin or activin are screened for utility in immunoassays for inhibin or activin by measuring competitive immunodisplacement of labelled inhibin or activin of the native hormones from polyclonal antibody directed against the native hormones. Exemplary contemplated sequence variants of Inh β_A include Phe₂₂₈->Ile or Leu; Gln₂₂₈->Asp or Lys; Trp₂₂₈->Tyr or Phe; Trp₂₁₈->Tyr or Phe; Ile₂₁₁->Phe or Val; Tyr₂₁₇->Trp or Thr; His₂₁₄->Lys; Ala₂₁₁->Ser; Asn₂₂₈->Gln, Tyr or His; Tyr₂₁₁->Thr or Asp, Phe₂₂₈->Tyr (a TGF- β/β_A intrachain hybrid); His₂₂₈->Asp; His₂₂₈->Lys (a β_A/β_B hybrid); Phe₂₂₈->Tyr; Val₂₂₄->Phe; Val₂₂₄->Leu; Tyr₂₂₁->Thr; Tyr₂₂₁->Trp; Asn₂₂₈->Gln, His or Lys; Ile₂₁₁->Leu or Thr; Met₂₂₈->Leu or Ser; Val₂₂₈->Phe, Glu, Thr or Ile. Comparable modifications are made in the human β_B chain. For example, Inh β_A contains a phenylalanyl residue at position 302, and Inh β_B also contains a phenylalanyl residue at a homologous position (264, Fig. 9) when aligned in the same fashion as is shown for porcine β_B in Fig. 4A. Thus, since the Phe₂₂₈ residue of β_A is described above as substituted by isoleucine or leucine, the Phe₂₂₈ of β_B is substituted with the same residues.

A factor in establishing the identity of a polypeptide as inhibin, activin or an inhibin variant is the ability of antisera which are capable of substantially neutralizing the hormonal activity of mature inhibin or activin to also substantially neutralize the hormonal activity of the polypeptide in question. However it will be recognized that immunological identity and hormonal activity are not necessarily coextensive. For example, a neutralizing antibody for inhibin may not bind a candidate protein because the neutralizing antibody happens to not be directed to specifically bind a site on inhibin that is critical to its activity. Instead, the antibody may bind an innocuous region and exert its neutralizing effect by steric hinderance. Therefore a candidate protein mutated in this innocuous region might no longer bind the neutralizing antibody, but it would nonetheless be inhibin in terms of substantial homology and biological activity.

It is important to observe that characteristics such as molecular weight, isoelectric point and the like for a native or wild type mature inhibin or activin obtained from follicular fluid or other tissue sources are descriptive only for the native form. Variants contemplated by the foregoing definition will include other polypeptides which will not exhibit all of the characteristics of native analogue. For example, inhibin derivatives like the insertion mutants, deletion mutants, or fusion proteins described above will bring inhibin outside of the molecular weight established for the corresponding native inhibin because fusion proteins with mature inhibin or proinhibin itself as well as insertion mutants will have a greater molecular weight than native, mature inhibin. On the other hand deletion mutants of native, mature inhibin will have a lower molecular weight. Finally, post-translational processing of preproinhibin chains in heterologous cell lines may not be accomplished with the fidelity exercised by the homologous host cell, thereby resulting in some variation in the amino termini of the α and/or β chains. This variation may be encountered as residual prosequence remaining with the mature protein, or the loss of several mature residues that are cleaved off with the prosequence. The same is true with processing of the preprotein in heterologous recombinant cells.

Covalent modifications of inhibin, activin or prodomains are included within the scope hereof and include covalent or aggregative conjugates with other chemical moieties. Covalent derivatives are prepared by linkage of functionalities to groups which are found in the inhibin amino acid side chains or at the N- or C-termini, by means known in the art. For example, these derivatives will include: aliphatic esters or amides of the carboxyl terminus or residues containing carboxyl side chains, e.g., aspartyl residues; O-acyl derivatives of hydroxyl

group-containing residues such as seryl or alanyl; and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g. lysine or arginine. The acyl group is selected from the group of alkyl-moieties (including C3 to C10 normal alkyl), thereby forming alkanoyl species, and carbocyclic or heterocyclic compounds, thereby forming aroyl species. The reactive groups preferably are difunctional compounds known *per se* for use in cross-linking proteins to insoluble matrices through reactive side groups, e.g. m-Maleimidobenzoyl-N-hydroxy succinimide ester. Preferred derivatization sites are at histidine residues.

Covalent or aggregative derivatives of mature inhibin, activin or prodomain sequences are useful as reagents in immunoassay or for affinity purification procedures. For example, inhibin or prodomain is insolubilized by covalent bonding to cyanogen bromide-activated Sepharose by methods known *per se* or adsorbed to polyolefin surfaces (with or without glutaraldehyde cross-linking) for use in the assay or purification of anti-inhibin or anti-prodomain antibodies or cell surface receptors. Inhibin or a prodomain sequence also is labelled with a detectable group, e.g., radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates or conjugated to another fluorescent moiety for use in diagnostic assays, especially for diagnosis of inhibin or prodomain levels in biological samples by competitive-type immunoassays.

DNA which encodes the complete α and β chains of inhibin/activin is obtained by chemical synthesis, by screening reverse transcripts of mRNA from ovary, or by screening genomic libraries from any cell. It may be more efficient to simply synthesize portions of the DNA desired since screening is required to identify DNA to cDNA or genomic libraries that encode the α and β chains. Synthesis also is advantageous because unique restriction sites can be introduced at the time of preparing the DNA, thereby facilitating the use of the gene in vectors containing restriction sites otherwise not present in the native sequence, and steps can be taken to enhance translational efficiency as discussed above, without the need to further modify the DNA as by mutagenesis or the like. cDNA encoding the α or β chains is free of untranslated intervening sequences (introns) as well as free of flanking DNA encoding other proteins homologous to their source.

DNA encoding the α and β chains is obtained from other sources than porcine and human by (a) obtaining a cDNA library from the ovary of the target animal, (b) conducting Southern analysis with labelled DNA encoding porcine or human α and β chains or fragments thereof (generally, greater than 100 bp) in order to detect clones in the

cDNA library that contain homologous sequences. -
 (c) analyzing the clones by restricting enzyme analysis and nucleic acid sequencing so as to identify full-length clones and, if full length clones are not present in the library, recovering appropriate fragments from the various clones and ligating them at restriction sites common to the clones to assemble a clone encoding the full-length molecule. As shown *infra*, any sequences missing from the library can be obtained by the 3' extension on ovarian mRNA of synthetic oligodeoxynucleotides complementary to cDNA identified by screening the library, or homologous sequences are supplied from known animal cDNAs. This is particularly useful in constructing pre or prepro inhibin sequences to facilitate processing of preproinhibin to mature inhibin from the desired species.

Porcine and human ovarian cDNA libraries initially were probed for DNA encoding inhibin sequences using labelled oligonucleotides whose sequence was based on the partial amino acid sequence determined from analysis of purified porcine inhibin or, in the case of human cDNA, porcine cDNA probes. However, once having described cDNA encoding human and porcine inhibin and prodomains, one skilled in the art would realize that precisely hybridizing probes can be prepared from the described sequences in order to readily obtain the remainder of the desired human or porcine gene.

Nucleotide sequence analyses of identified porcine and human cDNA clones revealed the structures of the biosynthetic precursors of both forms of inhibin. Interestingly, the two inhibin chains are not derived from a single processed precursor. Instead, the two chains are translated from separate mRNAs and then assembled into the disulfide crosslinked two-chain molecule.

Figs. 1B and 2B and 6, 8 and 9 depict the DNA encoding the polypeptide chains constituting porcine and human preproinhibin and preproactivin. Obviously, degenerate codons may be substituted for those disclosed in these figures where the same amino acid is encoded. The DNA of Figs. 1B, 2B, 6, 8 and 9 is mutated in order to encode the amino acid variants of the α and β chains described above. In particular, the prepro sequences are deleted and a start codon inserted immediately 5' to the mature chain in question so that the chain is expressed directly in recombinant culture. The DNA also is labelled, e.g. with radioactive phosphorous, and used to screen ovarian cDNA libraries from other species to identify α or β chain encoding DNA from such other species as is generally described above.

Covalent labelling of this DNA is accomplished with a detectable substance such as fluorescent group, a radioactive atom or a chemiluminescent group by methods known *per se*. The labelled DNA is then used in conventional hybridization assays. Such assays are employed in identifying vectors and transformants as described in examples *infra*, or for *in vitro* diagnosis such as detection of mRNA in tissues.

Lengthy sequences desirably are synthesized in host cells transformed with vectors containing DNA encoding them, e.g. inhibin or prodomain sequence. Vectors are used to amplify the DNA which encodes the chains, either in order to prepare quantities of DNA for further processing (cloning vectors) or for expression of the chains (expression vectors). An expression vector is a replicable DNA construct in which a DNA sequence encoding an α or β chain is operably linked to suitable control sequences capable of effecting their expression in a suitable host. Cloning vectors need not contain expression control sequences. Such control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites (for prokaryotic expression), and sequences which control termination of transcription and translation. The vector should include a selection gene to facilitate the stable expression of the desired polypeptide and/or to identify transformants. However, the selection gene for maintaining α and/or β chain expression can be supplied by a separate vector in cotransformation systems using eukaryotic host cells.

Vectors comprise plasmids, viruses (including phage), and integratable DNA fragments i.e., fragments that are integratable into the host genome by recombination. The vectors described herein for use in eukaryotic cell expression of inhibin α and/or β chains contain plasmid sequences for cloning in microbes, where the plasmid replicates autonomously from the host genome, but the DNA is believed to integrate into the eukaryotic host cell genome upon transformation. Similarly, bacillus vectors that genomically integrate by homologous recombination in bacillus also are useful. However, all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein.

Suitable vectors generally will contain replicon (origins of replication, for use in non-integrative vectors) and control sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or transfected with vectors containing inhibin α and/or β chain encoding DNA. Transformed host cells contain cloned DNA and, when transformed with an expression vector, also

express the α and/or β chains. The expressed polypeptides will be deposited intracellularly or secreted into either the periplasmic space or the culture supernatant, depending upon the host cell selected and the presence of suitable processing signals in the expressed protein, e.g. homologous or heterologous signal sequences.

DNA regions are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of secretory leaders, contiguous and in reading phase.

Suitable host cells are prokaryotes, yeast or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacilli*. Higher eukaryotic cells include established cell lines of mammalian origin as described below. A preferred host cell is *E. coli* 294 - (ATCC 31,446) although other prokaryotes such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), *E. coli* W3110 (ATCC 27,325), *Pseudomonas* species, or *Serratia Marcescans* are suitable.

Expression vectors for host cells ordinarily include an origin of replication (where extrachromosomal amplification is desired, as in cloning, the origin will be a bacterial origin), a promoter located upstream from the inhibin coding sequences, together with a ribosome binding site - (the ribosome binding or Shine-Dalgarno sequence is only needed for prokaryotic expression), RNA splice site (if the inhibin DNA contains genomic DNA containing one or more introns), a polyadenylation site, and a transcriptional termination sequence. As noted, the skilled artisan will appreciate that certain of these sequences are not required for expression in certain hosts. An expression vector for use with microbes need only contain an origin of replication recognized by the intended host, a promoter which will function in the host and a phenotypic selection gene, for example, a gene encoding proteins conferring antibiotic resistance or supplying an auxotrophic requirement. Inhibin DNA is typically cloned in *E. coli* using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., 1977 "Gene" 2: 95. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

Expression vectors, unlike cloning vectors, must contain a promoter which is recognized by the host organism. This is generally a promoter homologous to the intended host. Promoters most commonly used in recombinant DNA construction include the β -lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978, "Nature", 275: 615; and Goeddel et al., 1979, "Nature" 281: 544), a tryptophan (trp) promoter system (Goeddel et al., 1980, "Nucleic Acids Res." 8: 4057 and EPO Appl. Publ. No. 36,776) and the tac promoter [H. De Boer et al., 1983, "Proc. Nat'l. Acad. Sci. U.S.A." 80: 21-25]. While these are the most commonly used, other known microbial promoters are suitable. Details concerning their nucleotide sequences have been published, enabling a skilled worker operably to ligate them to DNA encoding inhibin in plasmid vectors (Siebenlist et al., 1980, "Cell" 20: 269) and the DNA encoding inhibin or its derivative. Promoters for use in prokaryotic expression systems also will contain a Shine-Dalgarno - (S.D.) sequence operably linked to the DNA encoding the inhibin, i.e., the S.D. sequence is positioned so as to facilitate translation. Generally, this means that the promoter and S.D. sequences located upstream from the second codon of a bacterial structural gene are substituted for the sequences of prepro inhibin located 5' to the mature α and/or β chains.

In addition to prokaryotes, eukaryotic microbes such as yeast cultures are transformed with inhibin-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other strains are commonly available and useful herein. Yeast vectors generally will contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the α and/or β chain, sequences for polyadenylation and transcription termination, and a selection gene. A suitable plasmid for expression in yeast is YRp7, (Stinchcomb et al., 1979, "Nature", 282: 38; Kingsman et al., 1979, "Gene", 7: 141; Tschemper et al., 1980, "Gene", 10:157). This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44078 or PEP4-1 (Jones, 1977, "Genetics", 85: 12). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., 1980, "J. Biol. Chem.", 255: 2073) or other glycolytic enzymes (Hess et al., 1968, "J. Adv. Enzyme Reg.",

Z: 149; and Holland *et al.*, 1978, "Biochemistry", 17, 4900), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hiltzman *et al.*, EP 73,657A.

Other yeast promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the inhibin or derivative coding sequences to provide termination and polyadenylation of the mRNA.

Cultures of cells derived from multicellular organisms are the preferred host cells herein because it is believed that expression of hormonally active inhibin or activin will only occur in such cells, with microbial expression resulting at most only in immunological cross-reactivity. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Propagation of vertebrate cells in culture *per se* has become a routine procedure in recent years - [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)].

Suitable host cells for expressing α or β chains in higher eukaryotes include: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); baby hamster kidney cells (BHK, ATCC CRL 10); chinese hamster ovary-cells-DHFR (described by Urlaub and Chasin, PNAS (USA) 77: 4218, - [1980]); mouse sertoli cells (TM4, Mather, J.P., Biol. Reprod. 23 : 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060652, ATCC CCL 51); rat hepatoma cells (HTC, M1, 54, Baumann, M., *et al.*, J. Cell Biol. 85: 1-8 - [1980]) and TRI cells (Mather, J.P. *et al.*, Annals N.Y. Acad. Sci. 383 : 44-68 [1982]).

The transcriptional and translation control sequences in vertebrate cell expression vectors preferably are provided from viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most preferably Simian Virus 40 (SV40). The early and late promoters of SV40 are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication - (Fiers *et al.*, 1978, "Nature", 273: 113). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the viral origin of the replication is included. Further, it is also possible to utilize the genomic promoters, control and/or signal sequences normally associated with the α or β -chains, provided each control sequences are compatible with and recognized by the host cell.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be obtained from SV40 or other viral (e.g. Polyoma, Adenovirus, VSV, or BFV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Rather than using vectors which contain viral origins of replication, mammalian cells are cotransformed with DNA encoding a selectable marker and DNA encoding the α and/or β chains. An example of a suitable selectable marker is dihydrofolate reductase (DHFR) or thymidine kinase. Such markers are proteins, generally enzymes that enable the identification of transformant cells, i.e., cells which had been competent to take up exogenous DNA. Generally, identification is by survival of transformants in culture medium that is toxic to untransformed cells or from which the cells cannot obtain a critical nutrient without having taken up the marker protein.

In selecting a preferred host mammalian cell for transfection by vectors which comprise DNA sequences encoding both inhibin and DHFR, it is appropriate to select the host according to the type of DHFR protein employed. If wild type DHFR protein is employed, it is preferable to select a host cell which is deficient in DHFR thus permitting the use of the DHFR coding sequence as marker for successful transfection in selective medium which lacks hypoxanthine, glycine, and thymidine (hgt⁻). An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, 1980, "Proc., Nat'l. Acad. Sci." (USA) 77: 4218.

On the other hand, if DNA encoding DHFR protein with low binding affinity for methotrexate - (MTX) is used as the controlling sequence, it is not necessary to use DHFR resistant cells. Because the mutant DHFR is resistant to MTX, MTX containing media can be used as a means of selection provided that the host cells are themselves MTX sensitive. Most eukaryotic cells which are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61). Preferably, transformants are first selected for neomycin resistance - (the transfection is conducted together with DNA encoding the neomycin resistance gene), followed by MTX amplification of the α and/or β chain expression as the case may be. See Kim *et al.*, "Cell" 42: 129-138 (1986) and EP 160,457A.

Other methods suitable for adaptation to the synthesis of α and/or β chains in recombinant vertebrate cell culture are described in M-J. Gething *et al.*, "Nature" 293: 620-625 (1981); N. Mantel *et al.*, "Nature" 281: 40-46; and A. Levinson *et al.*, EP 117,060A and 117,058A.

The inhibin α chain is expressed in recombinant cell culture with or without either of the β -chain molecules. Similarly, host cells are transformed with DNA encoding either or both of the mature β -chains. Based on analogy to TGF- β , the mature β -chains are capable of forming homodimers or β_A/β_B heterodimers upon expression in recombinant culture. These structures are not inhibin and will be referred to herein as β -chain dimers or activin. These are useful in the preparation of active inhibin, serving as sources of the β -chain, or are used as gel electrophoresis standards to detect the diversion into β -chain dimers of β -chains synthesized in α and β chain cotransformants. As will be seen in Example 4, this is not a hypothetical problem. Of course, the dimers are also useful in modulating reproduction as noted above.

β -chain hetero or homodimers are separated by *in vitro* unfolding of the individual chains followed by oxidative disulfide bond formation with the α -chain in accord with processes generally known *per se*. Preferably, however, in preparing mature inhibin the recombinant host is transformed with DNA encoding both the α and either of the β -chains. The intact hormonally active molecule is then assembled by the host cell *in vivo*, and it is thus unnecessary to combining the two chains by *in vitro* processing. The DNA encoding the α and β -chains is preferably located on the same vector, and under the control of the same promoter, but this is not essential.

Certain β -chain amino acid sequence variants identified in the screening procedure will not bind to pituitary cell surface receptors nor as a consequence will they exhibit hormonal activity. Such variants, when expressed as homodimers in recombinant cell culture, are useful in immunoassays for activin when they bear immunological epitopes cross-reactive with the native β -chain. In addition, such variants are coexpressed with DNA encoding hormonally active β -chain to yield a hybrid bearing native and variant β -chain. In this case the variant serves to stabilize the structure of the native β -chain. This form of β -chain heterodimer is useful, like the homodimer, in immunoassays for activin. It may also function as an activin antagonist.

The activin/inhibin β -chains also are coexpressed with TGF- β in order to produce β -chain/TGF- β hybrids. Vectors and methods for the expression of TGF- β are known. For example, see Derynck *et al.*, Human Transforming Growth Factor- β Complementary DNA Sequence and Expression in Normal and Transformed Cells "Nature" 316: 701-705 (1985). Contransformation of mammalian host cells by vectors bearing the TGF- β gene as described by Derynck *et al.* together with the β_A or β_B chains of activin/inhibin will result in secretion of a proportion of a β -chain/TGF- β hybrid dimers. This hybrid is useful in preparing TGF- β/β -chain immunogens or in immunoassays.

Inhibin, activin or prodomain sequences are recovered from transformed cells in accord with *per se* known procedures. When a polypeptide is expressed in recombinant bacteria as a refractile body, the desired polypeptide is recovered and refolded by conventional methods. Alternatively, the culture supernatants from transformed cells that secrete activin or inhibin, preferably mammalian cells, are simply separated from the cells by centrifugation. Then the inhibin generally is purified by successive purification procedures that include heparin-Sepharose affinity chromatography, gel filtration and at least one and preferably several RP-HPLC (reverse phase high pressure liquid chromatography) steps using different conditions in the stationary phase and/or mobile phase. Prodomain sequences produced by *in vitro* synthesis will be purified by conventional methods.

The prodomain polypeptides that are preferred for use herein are recovered from the culture media of recombinant cells transformed to synthesize the α and/or β chains as appropriate for the desired prodomain. Specifically, they are recovered by separating the culture medium polypeptides on native electrophoresis gel, excising bands having the predicted molecular weight and thereafter purifying the eluted polypeptides further, for example by FPLC or HPLC, followed by amino acid sequence determination for the substantially homogeneous

separated polypeptides. Purified prodomain polypeptides then are used to raise antibodies, e.g., in rabbits, which when used in immunoaffinity purification will simplify the recovery of the prodomains.

In the preferred procedure for isolating porcine hormonally active inhibin, clarified transformant culture supernatant or cell lysate is first purified by heparin-Sepharose affinity chromatography, next by gel filtration on Sephacryl S-200 gel and then with four successive RP-HPLCs using different mobile phase gradients and/or derivatized silica supports. Preferably, stationary phases having relatively low hydrophobicity are used, with C3-C8 columns being preferred and C3-C5 and phenyl columns being particularly preferred. Solute specificity of the mobile phase is preferably adjusted by varying the concentration of an organic component, particularly acetonitrile. Although a single RP-HPLC fractionation significantly increases the purity relative to the gel-filtrated material, two or more, and preferably four, RP-HPLC purifications are generally performed subsequent to successive treatment by heparin-Sepharose chromatography and gel filtration. This method has been found to be adaptable to the purification of human inhibin from recombinant cell culture as well.

The first step of the purification is heparin-Sepharose affinity chromatography, in which the protein is adsorbed to the Sepharose-bound heparin moieties under application conditions, and the adsorbed inhibin material is recovered by 1M NaCl elution. This step greatly expedites the purification procedure for crude extracts because it allows a relatively large volume of a crude extract to be processed fairly rapidly while recovering an amount of protein exhibiting total inhibin activity equal to at least 90% of that of the crude extract.

For the detection of inhibin activity in the various column fractions, aliquots ranging from 0.01% to 0.1% by volume are removed, and after added 100 μ g human serum albumin in 100 μ l water, the solvents were evaporated in a Speed-Vac concentrator (Savant, Hicksville, N.Y.). The residue as redissolved in 3 ml 1% fetal bovine serum in HDMEM, filtered through a Millex-GS 0.22 μ m filter (Millipore Corp., Bedford, MA) and assayed in duplicate. To speed up the bioassays during the purification process, only basal inhibition of FSH secretion exerted by the inhibin activity is determined and plotted in the region where the inhibin proteins were expected to migrate in the chromatograms.

To perform the heparin-Sepharose affinity chromatography, cell debris is spun down in a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA.) using a JA-20 rotor at 10,000 rpm for 30 minutes. One half of the supernatant is

diluted in 10 times its volume by the addition of 0.01 M Tris-HCl containing 0.1 M NaCl, pH 7, in an Erlenmeyer flask and pumped simultaneously via silastic tubes (0.76 mm ID) into heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) columns (3.5 x 9 cm) by two Rabbit 4-channel peristaltic pumps (Rainin Instrument Co., Inc., Emeryville, CA) at 40 ml/hr per column. After all the fluid has been pumped through the heparin-Sepharose, the eight columns are washed simultaneously with 0.01 M Tris-HCl, pH 7, containing 0.1 M NaCl in the same manner. The adsorbed proteins with inhibin activity are removed by washing the eight columns simultaneously with 0.01 M Tris-HCl containing 1 M NaCl, pH 7, as above, and the wash is collected into fractions. The inhibin activity is monitored by the *in vitro* bioassay described above. The columns are regenerated by further washing with 2M NaCl in 0.01 M Tris-HCl, pH 7, and re-equilibrated with 0.01 M Tris-HCl containing 0.1 M NaCl for purification of remaining extract.

Next, the material is fractionated by gel filtration to separate proteins generally according to their molecular weights. The fractions having inhibin activity extracted by the heparin-Sepharose columns were pooled and dialyzed overnight to remove NaCl in a 28.8 mm cylinder diameter Spectrapor No. 3 membrane tubing with M, cutoff at 3,500 (Spectrum Medical Industries, Inc., Los Angeles, CA.) against 30% acetic acid. The retained fluid is centrifuged, as above, to remove a white precipitate, and the supernatant is divided into equal portions for applying to 5 x 100 cm Sephacryl S-200 superfine columns (Pharmacia Fine Chemicals, Piscataway, N.J.). Each column is eluted with 30% acetic acid at 20 ml for 22 min., and the column fractions are monitored by UV absorption at 280 nm and by bioassay.

The bioassay-positive protein from the S-200 columns is pooled and lyophilized. The lyophilized material is dissolved in 0.2N acetic acid (1 ml/ml) and filtered through a Millex-HA 0.45 μ m filter (Millipore Corp., Bedford, MA.). The filtrate is applied directly onto a 1 x 25 cm Vydac 5- μ m particle-size C4 column (The Separations Group Hesperia, CA.) and developed with a gradient of TEAP buffer. In the TEAP system, buffer A consists of 0.25 N triethylammonium phosphate pH 3, and buffer B is 80% acetonitrile in buffer A. After all the filtrate had been loaded, the column is washed with the aqueous buffer A until the UV absorption reached baseline. The fractions exhibiting inhibin activity are separated in a Beckman 332 gradient liquid chromatography system (Beckman Instruments, Inc., Berkeley, CA.) equipped with a Spectroflow 757 UV detector (Kratos Analytical Instruments, Ramsey, N.J.), a Soltec 220 recorder -

(Soltec Corp., Sun Valley, CA.) and a Redirac 2112 fraction collector (LKB Instruments, Inc., Gathersburg, MD). Zones of inhibin activity are detected by bioassay.

Inhibin protein containing the β_B chain is further purified free of inhibin containing the β_A species, if desired, by two more RP-HPLC steps. The first step uses a 1 x 25 cm Vydac 5- μ m-particle-size C4 column and a trifluoroacetic acid (TFA) buffer system and the second step employs a 1 x 25 cm Vydac 5- μ m - particle-size Phenyl column and the TEAP buffer system. In the TFA system, buffer A contains 1 ml trifluoroacetic acid in 999 ml water and buffer B is 1 ml trifluoroacetic acid in 199 ml water and 800 ml acetonitrile. The two inhibin species elute separately. Inhibin accumulated from a few batches was concentrated by RP-HPLC using a 0.46 x 25 cm Aquapore RF-300 10 μ m-particle-size column (Brownlee Labs., Santa Clara, CA.) and the TFA buffer system. Ordinarily, however, this purification step will not be used with cell culture supernatants from transformants with DNA encoding only the β_A or β_B chains.

Inhibin, activin, prodomain sequences or their variants are administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes e.g., with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. Intravenous administration in isotonic saline, phosphate buffer solutions or the like is suitable.

The polypeptide herein should be administered under the guidance of a physician, and pharmaceutical compositions will usually contain an effective amount of the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier. The dosage will vary depending upon the specific purpose for which the protein is being administered, and dosage levels in the range of about 0.1 to about 1 milligram per Kg. of body weight may be used when inhibin is administered on a regular basis as a male contraceptive.

Inhibin, activin, prodomain sequences or their variants desirably are administered from an implantable or skin-adhesive sustained-release article. Examples of suitable systems include copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman *et al.*, 1983, "Biopolymers" 22(1): 547-556), poly (2-hydroxyethyl-methacrylate) (R. Langer *et al.*, 1981, "J. Biomed. Mater. Res." 15: 167-277 and R. Langer, 1982, "Chem. Tech." 12: 98-105) ethylene vinyl acetate (R. Langer *et al.*, *id.*), or

poly-D-(+)-3-Hydroxybutyric acid (EP 133,988A). Such articles are implanted subcutaneously or are placed into contact with the skin or mucous membranes.

In order to simplify the Examples certain frequently occurring methods will be referenced by shorthand phrases.

Plasmids are designated by a low case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from publicly available plasmids or DNA in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. "Partial" digestion refers to incomplete digestion by a restriction enzyme, i.e., conditions are chosen that result in cleavage of some but not all of the sites for a given restriction endonuclease in a DNA substrate. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements as established by the enzyme suppliers were used. Restriction enzymes commonly are designed by abbreviations composed of a capital letter followed by other letters and then, generally, a number representing the microorganism from which each restriction enzyme originally was obtained. In generally, about 1 μ g of plasmid or DNA fragment is used with about 1 unit of enzyme in about 20 μ l of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis *et al.*, 1982, Molecular Cloning pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide gel electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn *et al.*, 1981, "Nucleic Acids Res." **9**: 6103-6114, and D. Goeddel *et al.*, 1980, "Nucleic Acids Res." **8**: 4057.

"Southern Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern analysis shall mean separation of digests on 1 percent agarose, denaturation and transfer to nitrocellulose by the method of E. Southern, 1975, "J. Mol. Biol." **98**: 503-517, and hybridization as described by T. Maniatis *et al.*, 1978, "Cell" **15**: 687-701.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or chromosomal integrant. Unless otherwise provided, the method used herein for transformation of *E. coli* is the CaCl₂ method of Mandel *et al.*, 1970, "J. Mol. Biol." **53**: 154.

"Ligation refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis *et al.*, *Id.* p. 90., may be used.

"Oligonucleotides" are short length single or double stranded polydeoxynucleotides which are chemically synthesized by known methods and then purified on polyacrylamide gels.

All citations are expressly incorporated by reference.

EXAMPLE 1

Isolation of Cloned Inhibin α -Subunit cDNAs

The strategy for identification of clones containing coding sequences for the porcine inhibin subunits was based on the "long-probe" approach, successful in some previous instances (Anderson

et al., 1983, "Proc. Nat. Acad. Sci. USA" **80**:6836-6842 and Ullrich *et al.*, 1984, "Nature" **309**:418-425). Briefly, a high-complexity cDNA library constructed in λ gt10 and derived from porcine ovarian mRNA by oligo-dT-primed cDNA synthesis was screened with a single 64-base-long synthetic oligodeoxynucleotide directed against the N-terminal amino acid sequence of the α -chain of porcine inhibin. It was found that the library is to be prepared from fresh ovarian tissue because the inhibin chain mRNA was apparently quite labile. Approximately 1 in 2,000 plaques hybridized with this probe, and sequence analysis of several hybridizing cloned cDNAs confirmed correct probe identification. This analysis revealed that none of the characterized cDNAs contained sufficient sequence information to predict the complete structure of the α -chain precursor protein. Rather than analyzing more clones from the same cDNA library, a second library was constructed by 3' extension on ovarian mRNA of a synthetic oligodeoxynucleotide complementary to a sequenced region encoding α precursor residues 60-64 (Fig. 1A). This library was screened with a suitable restriction fragment from a previously analyzed cDNA and yielded several isolates which specified the remainder of the DNA sequences encoding the N-terminal region of the α precursor. Completeness of the coding sequence was judged from the presence of a long reading frame which specifies the porcine α -chain peptide sequence and starts with a methionine codon preceded by an in-frame stop codon and followed by a hydrophobic sequence bearing the hallmarks of a signal peptide. The full sequences for the precursor protein and its cDNA are shown in Fig. 1B. The complete protein including signal peptide has an Mr of ~40K consisting of 364 amino acids, of which the C-terminal 134 (Mr ~14.5K) constitute the porcine inhibin α -chain. There are several Arg-Arg sequences in the proregion of the precursor, one of them directly preceding the α subunit. We believe that this latter pair of basic residues is the processing site for the proteolytic release of the α peptide. The deduced precursor sequence predicts two N-linked glycosylation sites, one within the α chain proper.

In addition to the coding region, the cDNA sequence contains a 3'-untranslated sequence of 167 nucleotides, including the canonical AATAAA polyadenylation signal, and a 5'-untranslated region, the proper length of which is presently unknown.

The detailed method was as follows:

Polyadenylated mRNA was prepared from freshly frozen porcine ovaries (Kaplan *et al.*, "J. Biochem." **183**: 181-184) An oligo-dT-primed cDNA library of $\sim 8 \times 10^6$ clones in λ gt10 (Huyhn *et al.*, 1984, DNA Cloning Techniques, Ed. D. Glover)

was prepared from 5 μ g polyA⁺ mRNA as described by Wood *et al.*, "Nature" 312: 330-337 - (1984), except that the *Eco*RI adaptors used had the sequence 5'-AATTCATCTCGAGACGC-3' 3'-GTGAGCTCTGCG-5'P.

Approximately 1 x 10⁶ unamplified cDNA clones were screened with 5 α -subunit oligonucleotide 5'-ACCGCCCTTTGCCTTGGCCT-TGGTCCCCTGCTGCTCTGAGACTGCTGCAGAGACCTCCTGAGG-3', based on the amino acid sequence underlined in Fig. 1B. Hybridization was carried out with the phosphorylated ³²P-labelled probe in 5xSSC, 40% formamide at 37°C. Filters were washed at 50°C with 1xSSC, 0.1% SDS. Approximately 500 hybridization positive clones were obtained, twelve of which were purified and examined for insert size. The *Eco*RI inserts of five of these (λ PIN- α 2, - α 5A, - α 5, - α 8, - α 10) were subcloned into M13 derivatives (Messing *et al.*, 1981 "Nucl. Acids Res." 9:308-321) and sequenced by the dideoxy chain termination method of Sanger *et al.*, "Proc. Nat. Acad. Sci. USA" 74:5463-5467 - (1977). A specifically primed library was prepared by priming 5 μ g of polyA⁺ mRNA with the oligonucleotide 5'-CCCCACAGCATGTCTT-3' - (complementary to nucleotides 248-263) and subsequent cloning into λ gt10. Approximately 2x10⁶ clones of the 1x10⁶ clones obtained were screened with the 5' 100bp *Eco*RI-*Bam*HI fragment prepared from λ PIN- α 2. Twelve of the 170 hybridization positive clones obtained were purified and two (λ PIN-S12s, -S4s) were sequenced by the dideoxy method. The complete nucleotide sequences of the α -subunit cDNAs were obtained by subcloning various restriction fragments with the different λ isolates into the M13 phage derivatives. Compressions were resolved by the use of deoxyinosine mixes in combination with the *E. coli* single stranded binding protein (Pharmacia).

Isolation of Cloned Inhibin β Subunit cDNAs

The cDNA sequences encoding the precursors of the inhibin β subunits were obtained from the same cDNA libraries used for the α subunit. Overlapping cDNA clones were isolated by screening first with single long synthetic oligodeoxynucleotide probes based on the two N-terminal β subunit sequences and subsequently with suitable restriction fragments derived from characterized cDNA clones which served as probes for "walking" in both 5' and 3' directions (Fig. 2A).

In more detail, approximately 2x10⁶ oligo-dT primed ovarian cDNA clones were screened with the 5' end labelled β_A oligonucleotide, 5'-AAGAAG-CAGTTCTTTGTGTCCTTCAAGGACATTGGCTGG-AATGACTGGATCATTGC-3' based on the amino

acid sequence of residues 321-339. Five hybridization positives were obtained, of which three proved to contain β_A coding sequences (λ PIN- β_A 2, - β_A 4, - β_A 8). A 5', end 154 bp *Eco*RI-*Hind*III (nucleotides 158-297) fragment and a 3' end 213 bp *Eco*RI-*Pst* fragment (nucleotides 1679-1892) derived from λ PIN β_A 2 were used to screen 2x10⁶ oligo-dT primed cDNA clones and 2x10⁶ clones from the α -chain specifically primed library. Out of the sixteen clones analyzed in detail two were found to have longer 5' ends (λ PIN- β_A 5s, - β_A 22) and one clone λ PIN- β_A 21 contained the entire 3'-untranslated region. Porcine inhibin β_B subunit cDNA clones were isolated by screening 2x10⁶ clones from the specifically primed library with the β_B oligonucleotide 5'-

GGCCTGGAGTGTGATGGGAGAACCAACCTGCTCTGCCGCCAGGAATTTTCATCGATTTCAGGCT-3', which was based on the NH₂-terminal sequence described in Fig. 1A. Positive clones were further screened with the oligonucleotide inosine probe 5'-AATCTATIAAIAA C TG C -3' ("I" in this sequence stands for inosine), which covers all the possibilities in the non-coding strand for the amino acid sequence QQFFIDF. Two clones (λ PIN β_B -1s, -2s) were isolated and sequenced and found to code for the β_B subunit. A 230 bp *Eco*RI-*Sma* (nucleotides 21-251) fragment was isolated from λ PIN β_B -1 and used as a hybridization probe to screen 2x10⁶ oligo-dT primed cDNA clones. Two positives were obtained (λ PIN β_B -3,4). The nucleotide sequence of these overlapping clones was used to construct the sequence shown. All sequences were obtained by subcloning specific fragments into M13 phage vectors (Messing *et al.*, *op cit.*). The *Eco*RI restriction sites referred to above are all contained within the cDNA adaptor fragment, and do not refer to sequences present in the cDNA.

We noted that only very few clones from the oligo-dT-primed library (4 out of 2x10⁶) hybridized with the synthetic probe for the β -subunit of inhibin A. Although most of these proved correct by DNA sequence analysis, none contained a full 3'-untranslated region, as judged by the absence of a polyA homopolymer at their 3' ends. Absence of polyA tails suggested the existence of a very long 3'-untranslated sequence in this mRNA species and/or structural region(s) which prove difficult to copy by the polymerases used for library construction. Unexpectedly, a higher abundance (~10-fold) of inhibin β_A subunit coding sequences was found in the cDNA library made by specific priming on α -subunit mRNA. This library was screened with the synthetic probe for the β -chain of inhibin A on the subsequently refuted theory that the α precursor mRNA might also encode the β subunit. The high

abundance of inhibin β_A cDNA in this library was later traced to fortuitous complementarity of the specific α chain primer to a region in the 3'-untranslated portion of the corresponding mRNA.

Only four cloned cDNAs encoding the β subunit of inhibin B were found in our libraries. The sequence information obtained from these clones failed to reveal the complete structure of the corresponding precursor protein and its cDNA. The sequences of cDNAs and deduced protein structures for the precursors of the β subunits are compared in Fig. 2B. The nucleotide sequence of inhibin β_A subunit cDNA is 3.6 kb in length and contains an open reading frame for a protein of 425 amino acids (M_r ~46K), the C-terminal 116 residues of which represent the β subunit proper (M_r ~13K). This reading frame begins with a methionine codon followed by a sequence that codes for a characteristic signal peptide, the true length of which is believed to be 29 residues. The encoded β subunit is preceded by a string of 5 arginines at which it is presumably proteolytically cleaved from the precursor. Similar to the α subunit precursor, this β precursor contains several additional pairs of basic residues at which hitherto unknown biologically active peptide entities are believed to be released. It also contains one possible site of N-linked glycosylation in the proregion (Asn, residue 165).

The deduced protein sequence for the β subunit of inhibin B shows high homology with the β_A subunit sequence. 71 amino acid residues are identical and most changes are conservative in nature. Sequence homology, although of a lesser degree, is also found in the proregion of both β subunit precursors. Interestingly, an extremely purine-rich sequence rarely seen in coding regions but present in the cDNA encoding the inhibin β_A precursor and resulting in a curious amino acid sequence is not found in the cDNA which codes for the homologous β_B precursor. This results in a gap of 22 amino acid residues from the β_B precursor of inhibin when protein sequences are aligned for maximal homology. Such alignment also brings about a perfect match in the cysteine positions of both precursors (see Fig. 2B).

Northern Analysis of α and β chain Precursor mRNAs

Ovarian total and polyadenylated RNAs were analyzed by the Northern procedure using the sequenced cDNAs as probes to assess size and relative abundance of the mRNAs which encode the peptide subunits α and β and β_B of the heterodimeric inhibin molecule. Polyadenylated mRNA (2 μ g; lanes a, b, c, and f; 8 μ g; lane d) and total RNA (10 μ g; lanes e and g) were elec-

trophoresed into a formaldehyde 1.2% agarose gel and blotted onto nitro-c llulose filters. The following ³²P-labelled cDNA fragments were used as hybridization probes under stringent conditions. Lane a: 240 bp EcoRI-SmaI (nucleotides 134-371) from α subunit cDNA; b: 154 pb EcoRI-HindIII (nucleotides 158-297) from β_A subunit cDNA; c: 230 bp EcoRI-SmaI (nucleotides 21-251) from β_B subunit cDNA; d and e: EcoRI insert of λ PIN- α 2; f and g: EcoRI insert of λ PIN- β_A 5. Filters were washed for 2 hours with 3 changes of 0.1xSSC, 0.1% SDS at 60°C.

Analysis showed (Fig. 3) that α and β mRNAs are of different size and abundance, as indicated by results obtained from cDNA cloning. From their respective band intensities the α precursor mRNA is estimated to be at least of 10-fold higher abundance than the mRNA for the β_A precursor, and approximately 20-fold higher than the mRNA for the β_B precursor.

Using ribosomal RNAs as size standards, the α precursor mRNA, which is a single species, is 1500 nucleotides in length, a size in good agreement with the cloned cDNA sequence (Fig. 1B). β_A precursor mRNA sequences are represented by two main species of ~4.5 and ~7.2 kb in length. The relatively higher intensity of both species in polyadenylated than total RNA suggests that the 4.5 kb species does not represent 28S RNA which hybridized to the cDNA probe. Thus, the β precursor cDNA sequences shown in Fig. 2B are thought to represent the 4.5 kb mRNA, suggesting that the 5' untranslated region for the β_A mRNA is approximately 900 nucleotides long. The β_B precursor is encoded on one mRNA, of approximately 4.5 kb in size, which is present at roughly half the level of the two β_A mRNAs. Since the two β mRNAs are closely related, one can predict that both mRNAs have a similar structure and thus the β_B mRNA presumably possesses a long 5' and 3' untranslated region equivalent to that shown for the β_A mRNA. Choice of a different polyadenylation signal might explain the existence of the 7.2 kb species.

Homology To Transforming Growth Factor- β

The mature α and β inhibin subunits contain seven and nine cysteine residues respectively. Upon alignment of the cysteine residues it is apparent that the two subunits share a similar cysteine distribution and some sequence homology exists around these residues (Fig. 4), suggesting that both types of subunits derive from one ancestral gene. Surprisingly, significant homology was found between the β chain and the primary structure of human TGF- β recently determined. As outlined in Fig. 4, both peptides are of nearly equal length (inhibin β_A subunit, 116, β_B subunit 115;

TGFS, 116 residues) and show a strikingly similar distribution of their nine cysteine residues. Using this cysteine "skeleton" for alignment, the β_A and TGF- β sequences have an additional 31 residues in identical positions and show conservative changes in nine homologous places. Similar high homologies are seen upon comparison of the β_B and β -TGF. Some gaps were introduced for better alignment (Fig. 4). The overall homology reaches 35%, but approaches 60% in certain sections (cf. porcine inhibin β_A chain residues 11-45 and TGF residues 15-49), a very high degree of homology considering the difference in species. Interestingly, this homology extends beyond the termination codon for protein synthesis in the respective cDNAs. Thus, the cDNAs for TGF- β and both inhibin β subunits contain a highly G and C rich sequence in this region, and they also possess unusually long 5' and 3' untranslated regions.

One can discount the suggestion that the β subunit of inhibin is the porcine equivalent of human TGF- β , since there is almost absolute homology between human and murine β -TGFs. These findings strongly indicate that both inhibin subunits and TGF- β have a common ancestor and belong to one gene family. All three peptides are derived from similarly-sized precursors (M_r ~40K) where they occupy the C-terminal 110 or so residues and are released by proteolytic cleavage at pairs of arginines. They form homo- or heterodimers, and subunits in the biologically active complex are linked by disulfide bridges. However, there is little sequence homology between TGF- β and the β subunits in the pro parts of their precursors, although the regions comprising the odd residues which precede the β subunit and TGF peptides display limited but significant sequence relatedness.

EXAMPLE 2

Recombinant Synthesis of Porcine Inhibin

The plasmid used for recombinant synthesis of porcine inhibin was pSVE-P α B Δ Inh-DHFR. The procedure to construct this plasmid is shown in Fig. 5. This plasmid was constructed as follows:

pHBS348-E (EP 0073856A) was partially digested with *Eco*RI, blunted with *E. coli* DNA polymerase I (Klenow fragment) and the four dNTPs, ligated and the ligation mixture was transformed into *E. coli* in 294 (ATCC 31446). The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmids were screened for the loss of the *Eco*RI site preceding the SV40 early promoter. A plasmid having the site deleted is referred to as pHBS348-EII.

pHBS348-EII was digested with *Eco*RI and *Eco*RI to produce two fragments, fragment 1 containing the SV40 early promoter, pML-Amp^r sequences and the HBsAg 3' untranslated region and fragment 2 containing the HBsAg (hepatitis B antigen) coding sequences.

λ PIN β_A 5_s containing the coding region for the porcine inhibin β_A subunit was digested with *Eco*RI and *Sma*I and the 1335 bp fragment (fragment 3) containing the β_A coding region recovered by polyacrylamide gel electrophoresis. Fragment 1, recovered by agarose gel electrophoresis, was ligated to fragment 3 and the ligation mixture transformed into *E. coli* strain 294 (ATCC 31446). The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct DNA fragments. This plasmid is referred to as pSVE-p β_A Inh.

pHBS348-E (EP 0073856A) was partially digested with *Eco*RI, blunted with *E. coli* DNA polymerase I (Klenow fragment) and the four dNTPs, ligated to the synthetic oligonucleotide 5' GGTCGACC-3' containing the *Sal*I recognition site. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmids were screened for the presence of the extra *Sal*I restriction site. Plasmid DNA is prepared from this construction (pHBS348-ESalI).

λ PIN α -12s and λ PIN α -2 were digested with *Eco*RI and *Bam*HI. A 104 bp *Eco*RI-*Bam*HI fragment from λ PIN α -12s containing the 5' coding region and a 1246 bp *Eco*RI-*Bam*HI fragment from λ PIN α -2 containing the middle and 3' coding region were recovered and ligated together. The ligation mixture was digested with *Eco*RI, the enzyme heat denatured, and the mixture ligated to *Eco*RI-digested pUC9 (BRL). Recombinants were selected and confirmed by restriction analysis. DNA was prepared from the correct plasmid (pPIN α).

pPIN α , containing the complete coding region for porcine α -inhibin was digested with *Nco*I and *Eco*RI, filled in by *Pol*(I)K in the presence of 4dNTP's, the 1280 bp fragment (fragment 4) was recovered by gel electrophoresis. pHBS348-ESalI was digested with *Sst*II and *Hind*III, filled in by *Pol*(I)K in the presence of 4dNTP's, and fragment 5 containing the PML-Amp^r region, SV40 early promoter and HBsAg 3' untranslated region was recovered by gel electrophoresis. Fragments 4 and 5 were ligated together and the ligation mixture used to transform *E. coli* 294 (ATCC 31446). Recombinants were selected by growing on Ampicillin media plates. The desired recombinant is called pSVE-P α Inh.

pHBS348-ESall was digested with Sall and HindIII and fragment 6 containing the pML-Amp^r and SV40 early promoter was recovered by gel electrophoresis. pFD II (EP 117,060A) was digested with Sal I and HindIII and fragment 7 was recovered which contains the normal mouse DHFR gene fused to the HBsAg 3' untranslated region. Fragments 6 and 7 were ligated, and the ligation mixture transformed into E. coli strain 294 (ATCC 31446). The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct DNA fragments. This construction is referred to as pFDII-Sall.

pSVE-P α Inh was digested with Sall and fragment 8 was recovered which contains the SV40 early promoter and the α -inhibin coding region fused to the HBsAg 3'-untranslated region. pFDII-Sall was digested with Sall and fragment 9 containing the SV40 early promoter and the mouse DHFR coding region linked to the HBsAg 3'-untranslated region was recovered. pSVE- β _AInh was linearized by Sall digestion and ligated to fragments 8 and 9 in a three part ligation. The ligation mixture was transformed into E. coli strain 294 (ATCC 31446). The transformed culture is plated on ampicillin media plates and resistant colonies selected. Transformants were screened for the presence of fragments 8 and 9 in the correct orientation such that transcription from the three SV40 early promoters will proceed in the same direction. This final plasmid is designated pSVE-P α β _AInh-DHFR.

Plasmid pSVE-P α β _AInh-DHFR was transfected into DHFR deficient CHO cells (Urlaub and Chasin, 1980, PNAS 77,4216-4220). However, any DHFR mammalian host cell is suitable for use with this plasmid. Alternatively, any mammalian host cell is useful when the host cell is cotransformed with a plasmid encoding neomycin resistance, and transformants identified by their ability to grow in neomycin-containing medium.

The transfected CHO cells were selected by culturing in 15 HGT⁻ medium. The cells were allowed to grow to confluency in 15cm diameter plates. The cells thereafter were cultured in serum free medium for 48 hours prior to harvest. 50ml of supernatant medium was lyophilized after the addition of 100mg human serum albumin. The residue was redissolved in 3ml 1% fetal bovine serum in HDMEM (GIBCO Laboratories, Santa Clara, CA), filtered through a Millex-GS 0.22mM filter (Millipore Corp., Bedford, MA) and assayed in duplicate.

The inhibin hormonal activity in the transformant supernatants was determined by an *in vitro* bioassay using rat anterior pituitary monolayer culture, Vale, W. *et al.* *Endocrinology*, 91, 562-572 - (1972). In brief, 21-day-old female rat anterior

pituitaries were collected, enzymatically dispersed and plated in 10% fetal bovine serum in HDMEM - (GIBCO Laboratories, Santa Clara, CA) into 24-well tissue culture plates (Falcon Plastic, Oxnard, CA) on day 1. On day 2, the medium was changed to 1% fetal bovine serum in HDMEM, and the transformant medium sample was added. Incubation was continued for another 48 hours. The monolayer medium was then harvested, and the LH and FSH contents were determined by radio-immunoassay (RIA) using materials provided by The Pituitary Hormone Program of NIADDKD. In this assay, the inhibin-containing CHO cell culture inhibits the basal release of FSH but not LH, as compared to control pituitary cells that received the incubation medium only. The amount of porcine inhibin detected in transformant supernatants was 20 ng/ml and exhibited a dose response curve parallel to that obtained with pure porcine ovarian inhibin.

Immunological cross-reactivity is assayed by a sandwich-type radioimmunoassay. Rabbit antisera are raised against purified porcine follicular inhibin by s.c. immunization of rabbits with the porcine inhibin in Freund's complete adjuvant. The presence of anti-inhibin in the antiserum is detected by incubation of the antiserum with purified porcine inhibin and assaying for the formation of an immune complex by conventional techniques, e.g. gel filtration. An aliquot of the antisera is coated onto goat-anti-rabbit IgG precoated polystyrene test tubes. The recombinant culture supernatant or extract is diluted into phosphate buffered saline and added to the coated tubes, incubated overnight and washed. Another aliquot of the rabbit antiserum is added to the test tubes, incubated and washed. Radiolabeled goat antirabbit IgG is added to the tubes, incubated and unbound goat antiserum removed by washing. The recombinant produced inhibin cross-reacts with the rabbit antiserum, as evidenced by bound counts on the test tubes which exceed those of controls incubated with culture medium or extracts from untransformed host cells.

EXAMPLE 3

Construction of Human Inhibin Vector and Expression of Human Inhibin in Recombinant Cell Culture-1

Expression of human inhibin $\alpha\beta$ _A facilitated by the discovery that the mature porcine and human β _A chains are identical. Thus, construction of a vector for the expression of human inhibin can proceed from plasmid pSVE- β _AInh from Example 1, which contains the porcine β _A-encoding cDNA.

A λ gt 10 library of human ovarian cDNA made from 10 μ g of ovarian mRNA was subjected to Southern analysis using radiophosphate labelled porcine cDNA encoding α , β_A and β_B chains. λ HIN α -2 was identified as containing coding regions for the human α inhibin chain. The prevalence of hybridizing clones in the case of human α inhibin was considerably less than that found for porcine α inhibin, on the order of 1 in 100,000 human clones hybridized to the 685 bp SmaI fragment of the porcine cDNA for α inh. The β chain clones were also rare, with the β_B clones being present at about 3 times the level of β_A (1 and 3 out of about 1,000,000 clones, respectively). None of the β chain clones were full length. They were supplemented with a primed cDNA library and assembled generally as described above for the porcine cDNA. The λ inserts were recovered by EcoRI digestion.

Plasmids pHIN α -2 is digested with NcoI and SmaI, and the 1049 bp 15 fragment (fragment 10) is recovered by gel electrophoresis. pPin α (Example 2) is digested with EcoRI and PvuII. The 98 bp fragment (fragment 11) is recovered by gel electrophoresis. Fragments 10 and 11 are ligated to

adaptor I 5'-

CTGCTCCTCTTGCTGTTGGCCCCACGGAGTGGG-
GCATGGCTGCCAGGGCCCGGAGCTGGACC-3', in combination with adaptor II which is the complement of adaptor I. The resulting 1208 bp fragment - (fragment 12) is treated with Klenow fragment of Pol(I) and the 4 dNTP's and ligated to: pHBS348-ESall which has been restricted with HindIII and SacIII and blunt-ended as described in Example 1. Alternatively, pPin α was digested with EcoRI and HpaII with the fragment encoding upstream from the Hpa II site (that is, the first 21 residues of the porcine sequence) being recovered. The adaptor used in this alternative approach was

5'-CGGAGCTCGACC 3'
3'-CTCGAGCTGG 5'.

A plasmid pSVE-H α inh having the correct orientation of fragment 12 is identified by sequence analysis of transformants. This construction (pSVE-H α inh) thus contains the first 24 residues of the porcine signal sequence with the remainder being prepro human inhibin. Plasmid pSVE-H α inh is digested with SalI. The fragment containing the SV40 promoter and human inhibin sequence is ligated to fragment 9 and SalI digested pSVE- β_A inh (Example 2). This final plasmid designated pSVE-h $\alpha\beta_A$ inh-DHFR1 is transfected into DHFR-deficient CHO cells and selected as described in Example 2. The culture supernatant contains hormonally active human inhibin.

EXAMPLE 4

Construction of Human Inhibin Vector and Expression of Human Inhibin in Recombinant Cell Culture-II

This example is similar to Example 3 except that the pro sequence of human inhibin β_B was employed in the place of the porcine β_B prepro domain.

The lambda gt10 library of Example 3 yielded λ HIN α 2, as described in example 3, together with λ HIN β_A -5 and -14. The latter two phage were employed to construct the full length β_A coding cDNA by ligating the 311 bp EcoRI-Hind III fragment - (fragment 13) of λ HIN β_A -5 to the 1100 bp HindIII-HpaI fragment (fragment 14) of λ HIN β_A -14 and ligating this mixture in an EcoRI-SmaI digested mp18 vector (Biolabs). Clones were selected and screened for the appropriate sized insert. An mp18 vector containing the correct insert was treated with DNA polymerase(I) and the four dNTPs in order to render it double stranded, and thereafter digested with XbaI (which cleaves in the mp18 polylinker sequence), blunted with DNA polymerase I and the four dNTPs, and digested with EcoRI. A 1320 bp fragment (fragment 15) was ligated to the EcoRI-EcoRV fragment 1 from Example 2. This ligation mixture was used to transform *E. coli* 294 cells. Clones were screened by Southern Hybridization and confirmed by restriction analysis. The clone containing the hinh β_A coding sequence was designated pSVE-hum β_A inh. A plasmid containing the human β_A coding sequences and the human α -inhibin sequences together with the DHFR gene is constructed from plasmids pSVE-hum β_A inh, pSVE-H α inh and pFDIISall as outlined above. Specifically, the SalI fragments from pSVE-H α inh and pFDIISall which contain the human alpha inhibin and the DHFR genes were ligated with SalI digested pSVE-hum β_A inh and a clone containing all three genes was identified. This plasmid, designated pSVE-hum $\alpha\beta_A$ inh-DHFR2 was transfected into DHFR-CHO cells and selected by culture in ght⁻ medium. 24 clones were picked, grown to confluency in ght⁻ medium under conditions conventional for CHO cells for two days, allowed to rest for 2 more days and thereafter the culture media were assayed for inhibin and activin activity using the rat pituitary cell assay described above. 4 clones were found to secrete significant levels of human $\alpha\beta_A$ inhibin (h $\alpha\beta_A$ -8, 12, 14, and 18). The levels in the culture medium for each clone were, respectively, 125, 125, 200 and 250 ng/ml. Another clone (h $\alpha\beta_A$ -11) produced activin as the $\beta_A\beta_A$ homodimer, but no detectable inhibin, as determined by biological activity and the lack of a chain immunoreactivity in

the culture medium for this clone. Clone $\alpha\beta_A-16$ secreted only α chain and was devoid of activin or inhibin activity.

EXAMPLE 5

Recombinant Expression of Human Activin

As reported by Vale *et al.* (Id.) and Ling *et al.* (Id.), homodimers and heterodimers of the β chains A and/or B have the opposite effect of inhibin on the pituitary, introducing rather than inhibiting FSH secretion. These proteins, collectively termed activin, are made in α and β chain cotransformants as described in Example 4. However, somewhat less screening for an appropriate transformant is needed if the initial transfection is conducted with a vector or vectors that do not contain the α chain gene. A suitable vector is readily constructed from the above-described vectors by excising the α chain gene. Plasmid pSVE-hum β_A lnh from Example 4 is digested with *Sall* and ligated to fragment 9 (Example 2) containing the DHFR gene. The ligation mixture was used to transfect *E. coli* 294 cells and colonies selected on the basis of failure to hybridize to the α chain sequence but which did hybridize to the β chain DNA. A clone pSVE-hum β_A lnh-DHFR was identified from which the α chain DNA has been deleted. This clone is transfected into DHFR⁻ CHO cells as described above. Transformants are identified that secrete activin into the culture medium. Similarly, an expression vector containing a β_B coding sequence - (reconstituted by ligating DNA encoding the first 34 amino acids of human β_A to the remaining coding sequence of the human β_B chain) is readily constructed and cotransfected with pSVE-hum β_A lnh-DHFR to produce the heterodimer. The reconstructed human β_B gene also is used in the foregoing plasmids in order to produce $\alpha\beta_B$ -inhibin which, in the *in vitro* bioassay has essentially equivalent biological potency to the $\alpha\beta_A$ form of inhibin.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in the art may be made without departing from the scope of the invention which is set forth in claims appended hereto.

Claims

1. A method comprising (a) constructing a vector which includes nucleic acid encoding inhibin α chain and/or an inhibin β chain (b) transforming a host cell with the vector, and (c) culturing the transformed cell.
2. The method of claim 1 wherein the nucleic acid encoding the inhibin α chain and/or β chain is operably linked to a promoter recognized by the host cell and including the further step of recovering inhibin or a β chain dimer from the culture medium.
3. The method of claim 1 or 2 wherein the cell is a prokaryote.
4. The method of any preceding claim wherein the vector comprises nucleic acid encoding the prepro forms of both the inhibin α chain and an inhibin β chain.
5. The method of claim 1, 2 or 4 wherein the cell is a cell from a multicellular organism and hormonally active inhibin is produced.
6. The method of claim 2 wherein the promoter is a viral promoter.
7. The method of claim 6 wherein the promoter is an SV40 promoter.
8. The method of claim any preceding claim wherein mature porcine or human inhibin is recovered.
9. The method of any of claims 1 to 3 or 5 to 7 wherein the vector comprises nucleic acid encoding the prepro form of an inhibin β chain and a mature β -chain dimer is recovered free of the α -chain.
10. The method of claim 5 or 8 wherein the β chain is the β_A chain and the inhibin is present in a concentration greater than about 20 ng/ml in the culture medium.
11. The method of any preceding claim wherein the nucleic acid encodes an amino acid sequence variant of the inhibin α and/or β chain.
12. The composition comprising human or porcine inhibin which is completely free of unidentified or porcine proteins.
13. A composition comprising an α or β_B inhibin prodomain unassociated with native glycosylation.
14. A composition comprising a homodimer of mature inhibin β_A or β_B chains, which composition is free of the inhibin α chain.
15. A composition comprising a heterodimer of mature inhibin β_A with mature inhibin β_B , which composition is free of the inhibin α chain.
16. Non chromosomal DNA encoding inhibin α or an inhibin β chain.
17. The DNA of claim 16 which is free of intervening untranslated sequences.

18. The DNA of claim 16 or 17 which encodes porcine or human inhibin α or β chain.

19. The DNA of claim 16,17 or 18 which is labelled with a detectable moiety.

20. The DNA of claim 16,17,18 or 19 which encodes an amino acid sequence variant of the α or β chain.

21. A replicable vector comprising DNA encoding an inhibin α and/or β chain.

22. The vector of claim 21 wherein the DNA encodes porcine or human inhibin α and/or β chain.

23. The vector of claim 22 comprising a viral promoter operably linked to the DNA encoding the inhibin α and/or β chains.

24. The vector of claim 21,22 or 23 which contains DNA encoding both inhibin α and an inhibin β chain.

25. The vector of claim 21,22 or 23 which contains DNA encoding an inhibin β chain but not the inhibin α chain.

26. A host cell transformed with a replicable vector comprising DNA encoding the inhibin α and/or an inhibin β chain.

27. The cell of claim 26 which is a eukaryotic cell.

28. The cell of claim 26 or 27 wherein the DNA encodes porcine or human inhibin α and/or β chain.

29. A cell-free composition containing an inhibin α chain prodomain sequence that is free of mature α chain sequence.

30. A cell-free composition containing

a) a polypeptide comprising the inhibin β_A chain prodomain sequence HSAAPDCPSALALPKDVPNSQPEMVEAVKKHILNMLHL, PDVTQPVPKAALLNAIRKLHVGVKGVGENGYVEIEDDIG, AEMNELMEQTSEITFAESGTARKTLHFEISKEGSDLSVERAEVWFLKVPKANRTRT-KVTIRLFQQQKHPQGSLODTGEEAEVGLKGERSELLLSEKVVDA, STWHVFPVSSIQRLDQGKSSLDVRIACEQCQESGASLVLLG, or naturally occurring mammalian amino acid sequence variants thereof;

b) a polypeptide comprising the inhibin β_B chain prodomain sequence CTSCGGFRRPEELGRVDGDFLEAV, HILSRQLMRGRPNITHAVPKAAMV-TALRKLHAGKVREDGRVEIPHLDGHASPGADGQERVSEIISFAETDGLASSRVRLYFFISNEGNQNLFFVQASLWLYLKLPPYVLEKGS, VRVKVYFQEQGHG-

DRWNMVEKRVDLKRSWHTFPLTEAIQALFERGE, LNLVQCDSCQELAVVPVFDVPGEESSHRPFVVQ-ARLGDSRHRI, or naturally-occurring mammalian amino acid sequence variants thereof; or

c) a polypeptide free of the mature α chain amino acid sequence comprising the inhibin α chain prodomain sequences KVRALFLDALGPPAV-TREGGDPGV, HALGGFTHRGSEPEEEEDVSQAILFPATDAS-CEDKSAARGLAQEAEEGLFRYMFRPSQHTRSRO-VTSAQLWFHTGLDRQGTAAASNSSEPLL-GLLALSPGGPVAVPMSLGHAPPHWAVLHLATSAL-SLLTHPVLVLLRCPLCTCSARPE-ATPFLVAHTRTRPPSGGERA, or naturally-occurring mammalian amino acid sequences variants thereof.

31. A cell-free composition containing an inhibin β chain prodomain sequence.

32. To the composition of claim 31 wherein the β chain is the β_A chain and the composition is free of mature β_A chain sequence.

33. The composition of claim 31 wherein the β chain is the β_B chain and the composition is free of mature β_B chain sequence.

34. The composition of claim 30 wherein the variant is the corresponding porcine amino acid sequence.

35. The composition of any of claims 29 to 34 wherein the polypeptide is unaccompanied by native glycosylation.

36. The composition of any of claims 29 to 35 which is sterile and wherein the polypeptide further comprises an immunogenic polypeptide.

37. An antibody capable of binding a predetermined polypeptide of any of claims 29 to 36.

38. The composition of any of claims 29 to 36 wherein the polypeptide is conjugated to a detectable group.

39. The composition of claim 38 wherein the group is an enzyme, fluorophore or radioisotope.

40. The composition of any of claims 29 to 36, 38 or 39 which is insolubilized by non-covalent absorption or covalent cross-linking to a water insoluble support.

41. The composition of any of claims 29 to 36 or 38 to 40 further comprising a physiologically acceptable implantable matrix for controlled release of the polypeptide into the tissues of an animal.

Fig. 1A.

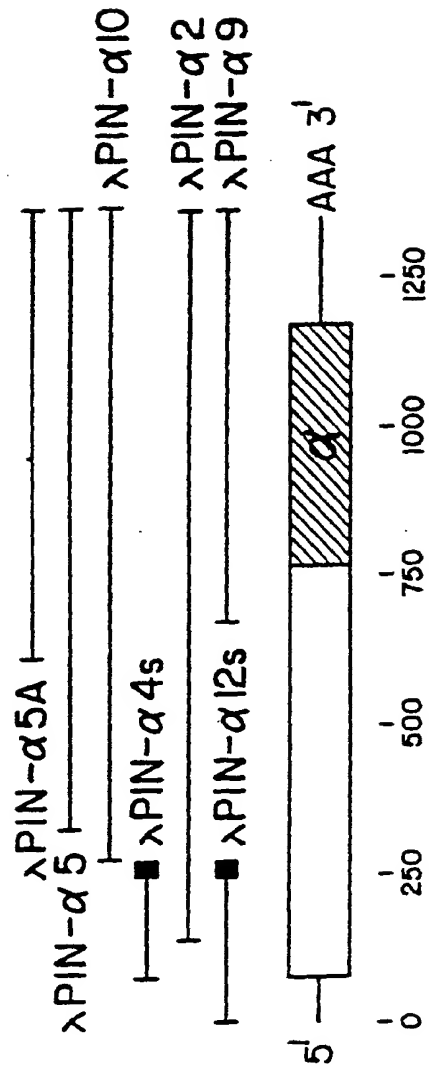


Fig. 1B(I)

	1	met trp pro gln leu leu leu leu leu leu ala pro	10
1	TGTGGGCAGACCCCTGACAGAAGGGSCACAGGGCTGGGTGTGGTTTACCGTTGGCAGGGCCAGGTGAGCT ATG TGG CCT CAG CTG CTC CTC TTG GTG GCC CCA		
108	arg ser gly his gly gln gln asp arg glu leu val leu ala lys val arg ala leu phe leu asp ala leu gly pro	20	40
	CGG AGT GGG CAT GGC CAG GGC CCG GAG CTG GAC CGG GAG CTT GTC CTG GCC AAG GTG AGG GCT CTG TTC CTG GAT GCC TTG GGA CCC	30	
198	pro ala val thr gly glu gly asp pro gly val arg arg leu pro arg arg his ala val gly gly phe met arg arg gly ser glu	50	70
	CCG GCA GTG ACT GGG GAA GGT GGG GAT CCT GGA GTC AGG CGT CTG CCC CGA AGA CAT GCT GTG GGG GGC TTC ATG CGC AGG GGC TCT GAG		
288	pro glu glu asp val ser gln ala ile leu phe pro ala thr gly ala arg cys gly asp glu pro ala ala gly glu leu ala arg	80	100
	CCC GAG GAG GAG GAT GTC TCC CAG GCC ATC CTT TTC CCG GCT ACA GGT GCC CGG TGT GGG GAC GAG CCA GCT GCT GGA GAG CTG GCC CGG	90	
378	glu ala glu glu gly leu phe thr tyr val phe arg pro ser gln his thr his ser arg gln val thr ser ala gln leu trp phe his	110	130
	GAG GCT GAG GAG GGC CTC TTC ACA TAT GTA TTC CGG CCG TCC CAG CAC ACA CAC GAG GTG ACT TCA GCT CAG CTG TGG TTC CAC	120	
468	thr gly leu asp arg gln gly met ala ala asn ser ser gly pro leu leu asp leu leu ala leu ser ser arg gly pro val ala	140	160
	ACG GGA CTG GAC AGA CAG GGG ATG GCA GCC GCC AAT AGC TCT GGG CCC CTG CTG GAC CTG CTG GCA CTA TCA TCC AGG GGT CCT GTG GCT	150	
558	val pro met ser leu gly gln ala pro pro arg trp ala val leu his leu ala ala ser ala leu pro leu leu thr his pro val leu	170	190
	GTG CCC ATG TCA CTG GGC CAG GCG CCC CCT CGC TGG GCT GTG CTG CAC CTG GCC GCC TCT GCC CTC CCT TTG TTG ACC CAC CCA GTC CTC	180	

Fig. 1B(II)

648 val leu leu leu arg **cys** pro leu **cys** ser ala arg pro glu ala thr pro phe leu val ala his thr arg ala arg pro pro
 738 GTG CTG CTG CTG CGC **TAT** CCT CTC **TAT** TCC **TGC** TCA GCC CGG CCC GAG GCC ACC CCC TTC CTG GTG GCC CAC ACT CGG GCC AGG CCA CCC
 828 ser gly gly glu arg ala arg ser thr ala pro leu pro trp pro trp pro trp ser pro ala ala leu arg leu leu gln arg pro pro glu
 918 AGC GGA GGG GAG AGG GCC CGA CGC TCC ACC CCT CTG CCC TGG CCT TGG TCC CCC GCC GCG CTG CGC CTG CAG AGG CCC CCG GAG
 1008 glu pro ala val his ala asp **cys** his arg ala ser leu asn ile ser phe gln glu leu gly trp asp arg trp ile val his pro pro
 1098 GAA CCC GCT GTG CAC GGC GAC **TGC** CAC AGA GCT TCC AAC ATC TCC TTC CAG GAG CTG GGC TGG GAC CGG TGG ATC GTG CAC CCT CCC
 1194 ser phe ile phe his tyr **cys** his gly gly **cys** gly leu pro thr leu pro leu ser val pro gly ala pro pro thr pro
 1312 AGT TTC ATC TTC CAC TAC **TGT** CAC GGG GGC **TGC** GGG CTG CCG ACC CTG CCC AAC CTG CCC CTG TCT GTC CCT GGG GCC CCC CCT ACC CCT
 1208 val gln pro leu leu leu val pro gly ala gln pro **cys** **cys** ala ala leu pro gly thr met arg ser leu arg val arg thr thr ser
 1312 GTC CAG CCC CTG TTG TTG CCA GGG GCT CAG CCC **TGC** GCT GCT CTC CCG GGG ACC ATG AGG TCC CTA CGC GTT CGC ACC ACC TCG
 1328 asp gly gly tyr ser phe lys tyr glu thr val pro asn leu leu thr gln his **cys** ala **thr** ile OC
 1338 GAT GGA GGT TAC TCT TTC AAG TAC GAG ACG GTG CCC AAC CTT CTC ACC CAG CAC **TGT** GCC **TGC** ATC TAA GGGTGTCCCGCTGGTGGCGGAGCTCCC
 1348 ACAGGCACCGCTGGAGGAAGGACAGATTCCACACCTCCCTTTCTTCGGCCTCTCGGCGCTGGAGGCTCCCTCCCTGTCGCCCTGTGTTCCCATGGGTAATGTGACAATAAACAGCAT
 1358 AGTCAGATGACTCGGTGCGCAAAAAA

Fig. 2A.

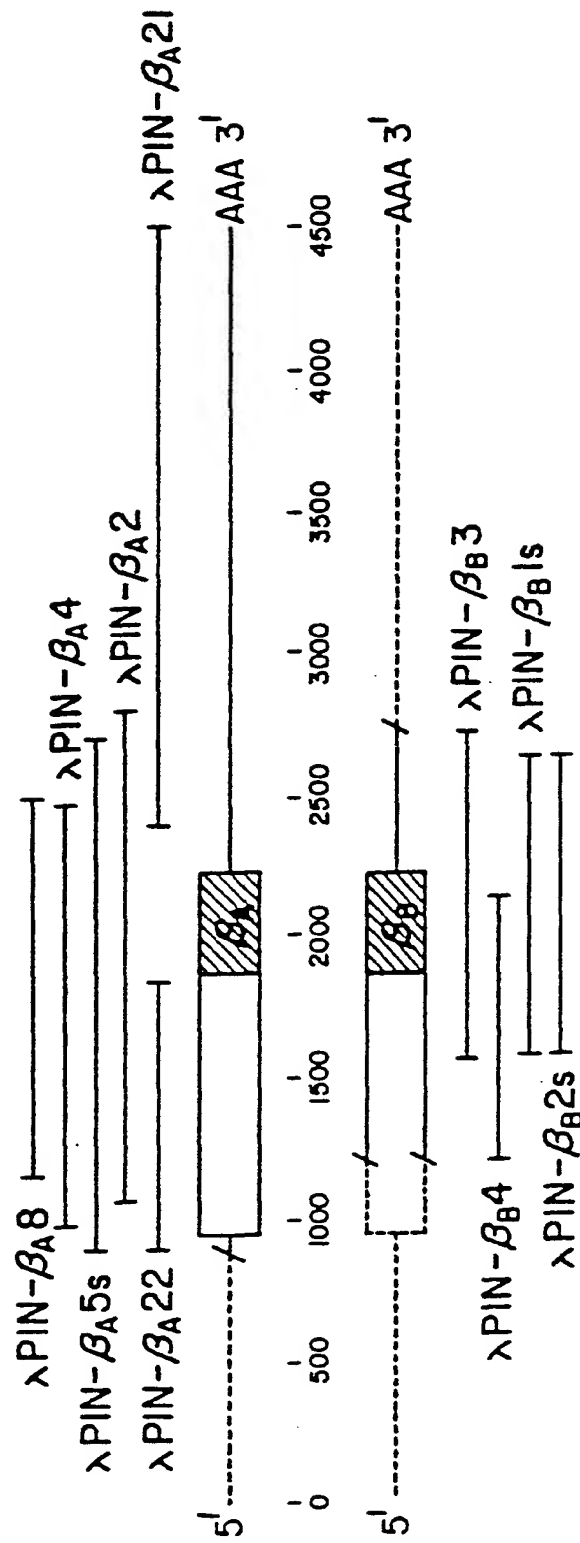


Fig. 2B(I)

[illegible]

Fig. 2B(II)

140 glu ile ser lys glu gly ser asp leu ser val val glu arg ala glu ile trp leu phe leu lys val pro lys ala asn arg thr arg
 448 GAG ATC TCC AAA GAG GGC AGT GAC CTG TCC GTG GAG CGC GGC GAA ATC TGG CTC TTC CTG AAA GTG CCC AAG GCC AAC CGG ACC AGG
 TTC ATC TCC AAC GAG GGT AAC CAG AAC CTG TTC GTG GTA CAG GCC AGT CTG TGG CTC TAC CTG AAG CTG CTG leu leu
 phe ile ser asn glu gly asn gln val val glu gln ala ser leu trp leu tyr leu lys
 150
 170 thr lys val ser ile arg leu phe phe gln gln gln arg arg pro gln gly ser ala asp ala gly glu glu ala glu asp val gly phe pro
 538 ACC AAA GTC TCC ATC CGT CTC TTT CAA CAG CAG AGG CGC CCG CAA GGC AGC GCG GAC GCA GGG GAG GAG GCG GAG GTG GGC TTC CCG
 --- CCT TAC GTT CTG GAG AAG GGC AGC CGG CGC --- AAG GTT CGG GTC AAG GTG TAC TTC CAG GAG CCG
 pro tyr val leu glu lys gly ser arg arg
 200 glu glu lys ser glu val leu ile ser glu lys val val asp ala arg lys ser thr trp his ile phe pro val ser ser ser file gln
 628 GAG GAG AAG AGC GAA GTG CTG ATT TCG GAG AAG GTG GTG GAT GCC CGG AAG AGC ACC TGG CAC ATC TTC CCG GTC TCC AGC AGC ATC CAG
 GGC CAC GGC GAC CGC TGG GAC GTG GTG GAG AAG CGT GTG GAC CTG AAG CCG AGC GGC TGG CAC ACC CTG CCG CTC ACC GAG GCC ATC CAG
 gly his gly asp arg trp asp val val glu lys arg val asp leu lys arg ser gly trp his thr leu pro leu thr glu ala file gln
 210
 220
 230 arg leu leu asp gln gly lys ser ala leu asp ile arg thr ala cys glu gln cys his glu thr gly ala ser leu val leu leu gly
 718 CGC TTG CTG GAC CAG GGC AAG AGC GCC CTG GAC ATC CGG ACT GCC TGC GAG CAG TGG CAC GAG ACC GGC GCC AGC CTG GTG CTG CTG GGC
 GCC CTG TTT GAG CGG GGC GAG CGG CGC CTC AAC CTG GAC GTG CAG TGC GAC GGC TGC CAG GAG CTG GCC GTG GTG CCC GTG TTT ---
 ala leu phe glu arg gly glu arg arg leu asn leu asp val gln cys asp gly cys gln gln leu ala val val pro val phe
 240
 250
 260
 270
 280
 290
 300
 310
 320
 330
 340
 350
 360
 370
 380
 390
 400
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 420
 430
 440
 450
 460
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 750
 760
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 780
 790
 800
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 820
 830
 840
 850
 860
 870
 880
 890
 900
 910
 920
 930
 940
 950
 960
 970
 980
 990
 1000

[illegible]

Fig. 2B(IV)

[illegible]

Fig. 3.

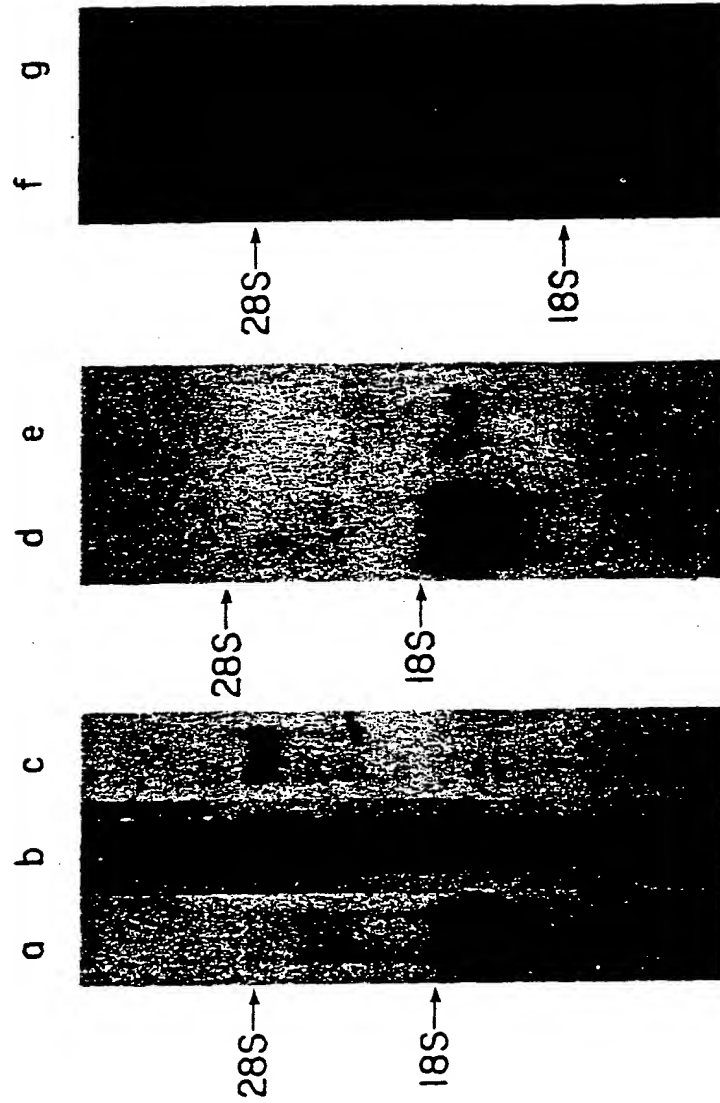


Fig. 4A.

p.β_A-Inh:
h.β-TGF:
p.β_B-Inh:

p.β_A-Inh:
h.β-TGF:
p.β_B-Inh:

p.βA-Inh:
h.β-TGF:
p.βB-Inh:

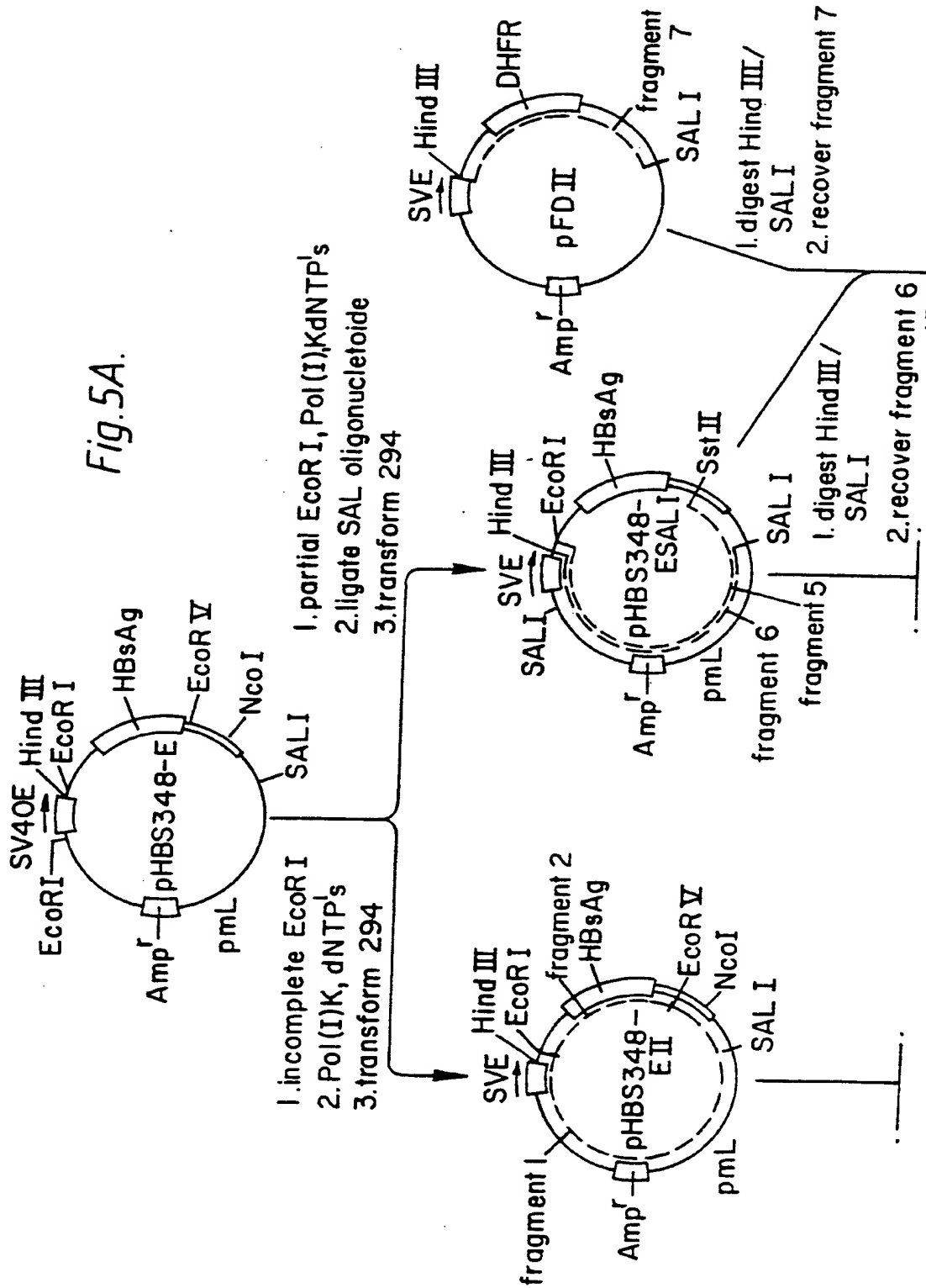
Fig. 4B.

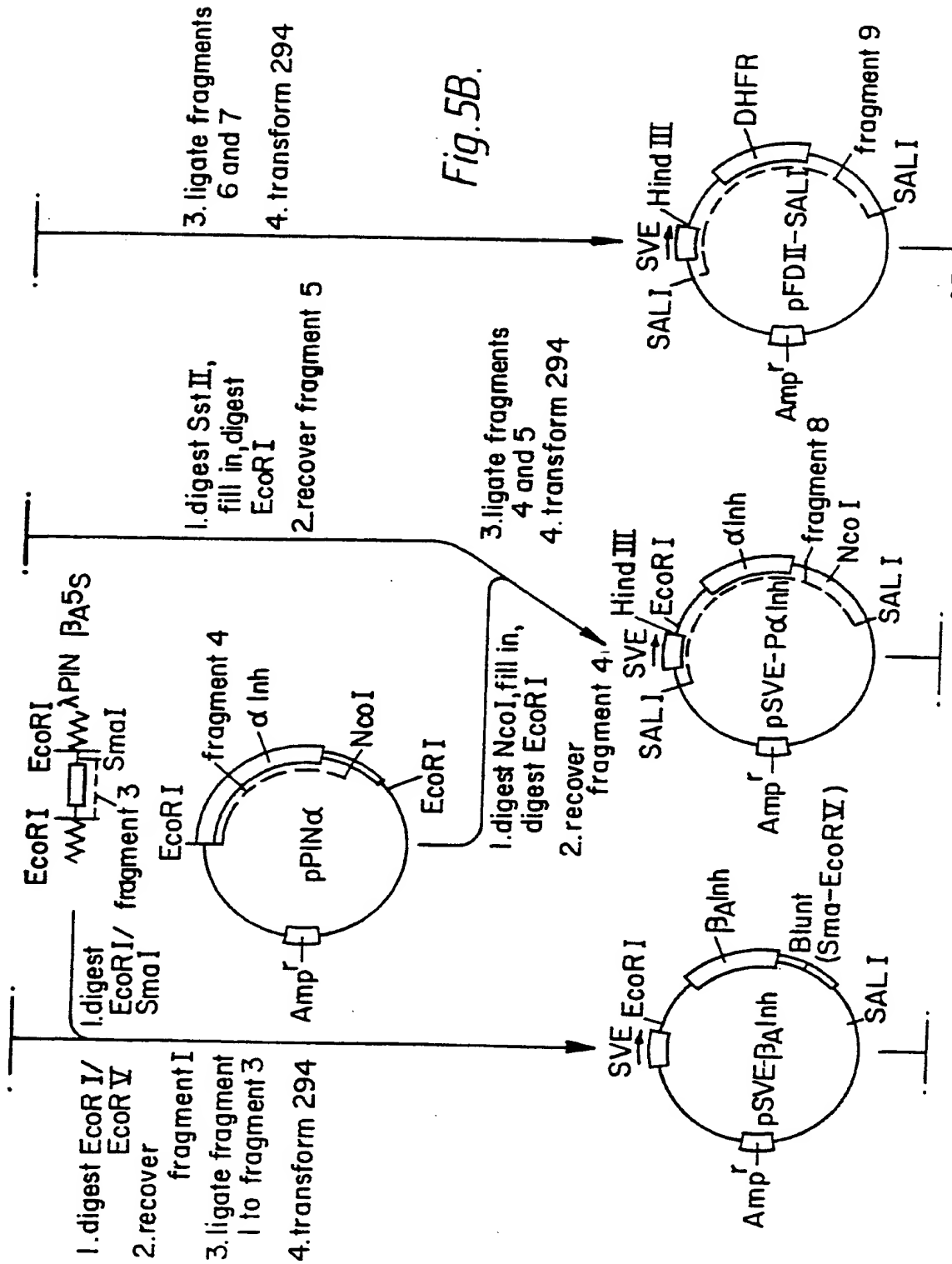
1
p.βA-Inh: GLECDGKVN^{*}I-CCKKQFFV^{*}SF^{*}KDIGW^{*}NDWI^{*}IA^{*}PSGYHANY^{*}39
p.α-Inh: RPPEEPA^{*}VHAD^{*}C^{*}HRASLN^{*}ISF^{*}QELGW^{*}DRWI^{*}VH^{*}PPSFI^{*}FHY^{*}58

40
p.βA-Inh: CEGEC^{*}PSHIAGTSGSS^{*}LSFHS^{*}TVINHYRMRGHSPFANLKS^{*}79
p.α-Inh: CHGGC^{*}GLPTLPNLPL^{*}SVPGAPPTPVQPLLVPGAQ^{*}---P^{*}94

80
p.βA-Inh: CC^{*}VPTKLRPMS^{*}MLYY^{*}--DDGQ^{*}-NIIKKD^{*}IQ^{*}NMI^{*}VEEC^{*}GS^{*}116
p.α-Inh: CC^{*}AALPGTMRSL^{*}RVRTTSDGGYS^{*}FKYETVP^{*}NLL^{*}TQH^{*}CAG^{*}I^{*}134

Fig. 5A.





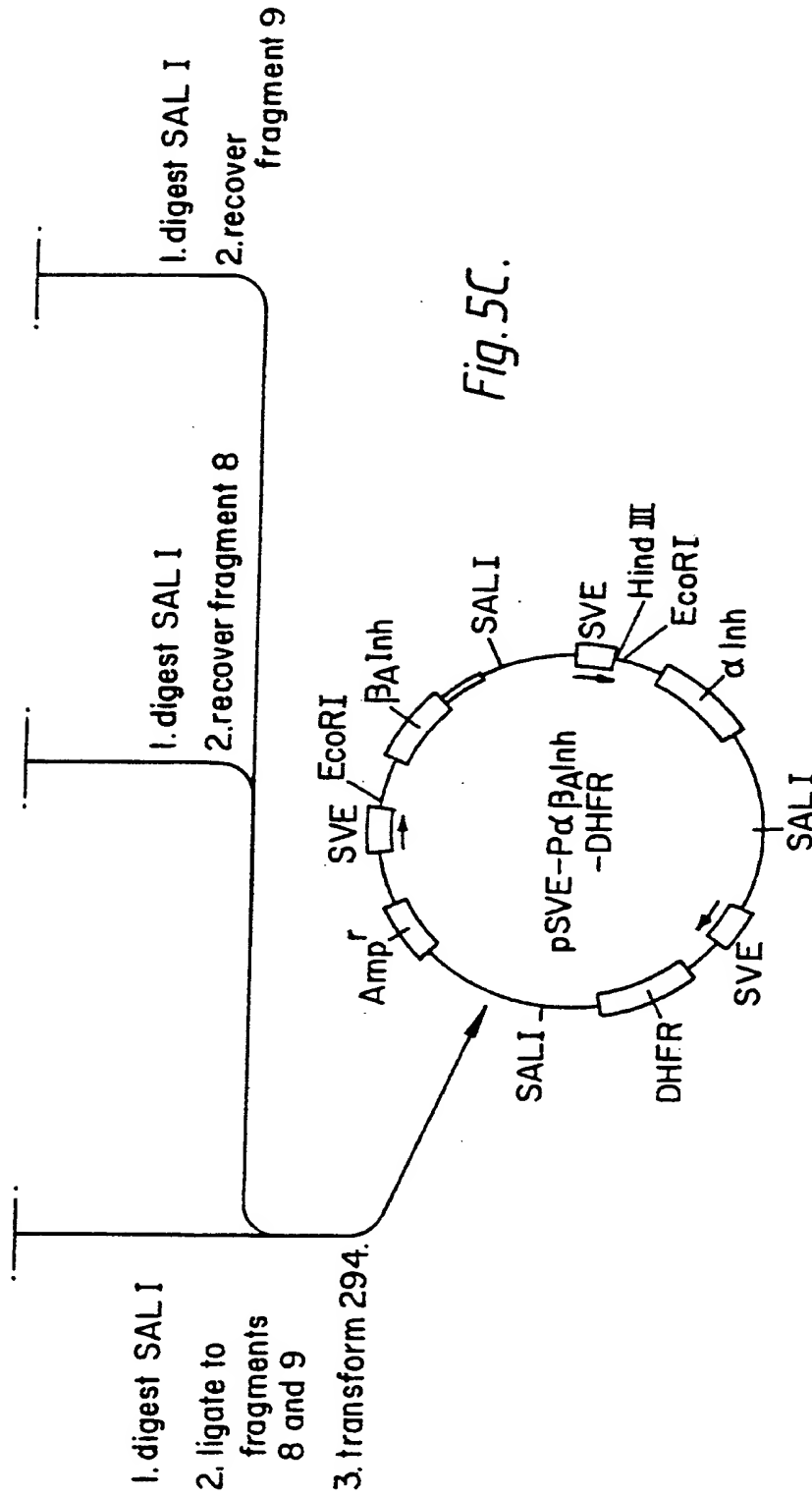


Fig. 5C.

0 222 491

-16
Gly Val Ser Ser Gln Gly Leu Glu Leu Ala Arg Glu Leu Val Leu Ala Lys Val Arg Ala Leu Phe Leu Asp

1 GT GGG GTC AGC AGC CAG GGG CTG GAG CTG GCC CGG GAA CTT GTT CTC GGC AAG GTG AGG GCC CTG TTC TTG GAT

-10 -1 +1

10
Ala Leu Gly Pro Pro Ala Val Thr Arg Glu Gly Gly Asp Pro Gly Val Arg Arg Leu Pro Arg Arg His Ala Leu

75 GCC TTG GGG CCC CCC CGG GTG ACC AGG GAA GGT GGG GAC CCT GGA GTC AGG CGG CTG CCC CGA AGA CAT GCC CTG

20 30

Gly Gly Phe Thr Hts Arg Gly Ser Glu Pro Glu Glu Glu Asp Val Ser Gln Ala Ile Leu Phe Pro Ala Thr

150 GGG GGC TTC ACA CAC AGG GGC TCT GAG CCC GAG GAA GAG GAG BAT GTC TCC CAA GCC ATC CTT TTC CCA GCC ACA

40 50

Asp Ala Ser [X] Glu Asp Lys Ser Ala Ala Arg Gly Leu Ala Gln Glu Ala Glu Glu Phe Arg Tyr Met

225 GAT GCC AGC [X] GAG GAC AAG TCA GCT GCC AGA GGG CTG GCG CAG GAG GCT BAG GAG GGC CTC TTC AGA TAC ATG

60 70 80

Phe Arg Pro Ser Gln Hts Thr Arg Ser Arg Gln Val Thr Ser Ala Gln Leu Trp Phe His Thr Gly Leu Asp Arg

300 TTC CGG CCA TCC CAG CAT ACA CGC AGC AGC CAG GTG ACT TCA GCC CAG CTG TGG TTC CAC ACC GGG CTG GAC AGG

90 100 130

110
Gln Gly Thr Ala Ala Ser Asn Ser Ser Glu Pro Leu Leu Gly Leu Ala Leu Ser Pro Gly Gly Pro Val Ala

375 CAG GGC ACA GCA GCC TCC AAT AGC TCT GAG CCC CTG CTA GGC CTG CTG GCA CTG TCA CCG GGA GGA CCC GTG GCT

120 150

Val Pro Met Ser Leu Gly Hts Ala Pro Pro His Trp Ala Val Leu His Leu Ala Thr Ser Ala Leu Ser Leu Leu


450 GTG CCC ATG TCT TTG GGC CAT GCT CCC CCT CAC TGG GCC GTG CTG CAC CTG GCC ACC TCT GCT CTC TCT CTG CTG

140 180



Thr His Pro Val Leu Val Leu Leu Leu Arg [X] Lys Pro Leu [X] Thr [X] Lys Ser Ala Arg Pro Glu Ala Thr Pro Phe

525 ACC CAC CCC GTC CTG GTG CTG CTG CTG GC [X] CAC CCC CTC CC [X] CAC GCG CCT GAG GCC ACG CCC TTC









Fig. 6B.







190 Leu Val Ala His Thr Arg Pro Pro Ser Gly Gly Arg Ala Arg Ser Thr Pro Leu Met Ser Trp
 600 CTG GTG GCC CAC ACT CGG ACC AGA CCA CCC AGT GGA GGG GAG AGA GCC CGC TCA ACT CCC CTG ATG TCC TGG



210 Pro Trp Ser Pro Ser Ala Leu Arg Leu Leu Gln Arg Pro Pro Glu Glu Pro Ala Ala His Ala Asn  His Arg
 675 CCT TGG TCT CCC TCT GCT CTG CCG CTG CAG AGG CCT CCG GAG GAA CCG GCT GCC CAT GCC AAC  CAC AGA

230 Val Ala Leu Asn Ile Ser Phe Gln Glu Leu Gly Trp Glu Arg Trp Ile Val Tyr Pro Pro Ser Phe Ile Phe His
 750 GTA GCA CTG AAC ATC TCC TTC CAG GAG CTG GGC TGG GAA CGG TGG ATC GTG TAC CCT CCC AGT TTC ATC TTC CAC

260 Tyr  His Gly Gly  Gly  Gln  Ser Phe  Gly  Leu His Ile Pro Pro Asn Leu Ser Leu Pro Val Pro Gly Ala Pro Pro Thr Pro
 825 TAC  CAT GGT GGT  GGG CTG CAC ATC CCA CCA AAC CTG TCC CTT CCA GTC CCT GGG GCT CCC CCT ACC CCA

280 Ala Gln Pro Tyr Ser Leu Leu Pro Gly Ala Gln Pro  Ala Ala Leu Pro Gly Thr Met Arg Pro Leu His
 900 GCC CAG CCC TAC TCC TTG CTG CCA GGG GCC CAG CCC  GCT GCT CTC CCA GGG ACC ATG AGG CCC CTA CAT

310 Val Arg Thr Thr Ser Asp Gly Tyr Ser Phe Lys Tyr Glu Thr Val Pro Asn Leu Leu Thr Gln His  Ala
 975 GTC CGC ACC ACC TCG GAT GGA GGT TAC TCT TTC AAG TAT GAG ACA GTG CCC AAC CTT CTC ACG CAG CAC  GCT

335  Ile OC*
 1050  ATC TAA GGGTGGGGGGTCTTCCTTCTTAATCCCATGGCTGGTGGCCACGCCCCACCATCATCAGCTGGGAGGAAGGCAGAGTTGGGAAATA

1146 GATGGCTCCCACTCCCTCCTTTACATTCTCTGCGCTATGGGCTACCGCTCCCGACCCCACTTCTATCTCAATAAAGAACACAGTGCATATG polyA

Fig. 7A.

pin.alpha	-30		+1	20
hin.alpha		MWPQLLLLLLAPRSGHGCQGPELDRELVLAKVRALFLDALGPPAVTGEGG		
		GVSSQGLELARELVLAKVRALFLDALGPPAVTREGG	*	
		*** *		
pin.alpha	40		60	
hin.alpha		DPGVRRLPRRHAVGGFMRRGSEPEEE-DVSQAILFPATGARGDEPAAGE		
		DPGVRRLPRRHALGGFTHRGSEPEEEEEVSQAILFPATDASCEDKSAARG	* ** ** *	
		* ** *		
pin.alpha	80		100	
hin.alpha		LAREAEEGLFTYVFRPSQHTHSRQVTSACLWFHTGLDRQGMAAANSSGPL		
		LAQEAEGLFRYMFRPSQHTRSRQVTSACLWFHTGLDRQGTAAANSSEPL	* * *	
		* * *		
pin.alpha	120		140	160
hin.alpha		LDLLALSSRGPVAVPMSLGGQAPPRWAVLHLAASALPLLTHPVLVLLLRCP		
		LGLLALSPGGPVAVPMSLGHAPPHWAVLHLATSALSLLTHPVLVLLLRCP	* * *	
		* ** *		

Fig. 7B.

pin.alpha	180	200	
hin.alpha	LCSCSARPEATPFLVAHTRARPPSGGERARRSTAPLP-WPWSPAALRL LQ		
	LCTCSARPEATPFLVAHTRTRPPSGGERARRST-PLMSWPWSPSALRL LQ	*	** *
			*
	220	240	260
pin.alpha	RPPEEPAVHADCHRASLNISFQELGWDRWIVHPPSFIFHYCHGGCGLPTL		
hin.alpha	RPPEEPAAHANCHRVALNISFQELGWERWIVYPPSFIFHYCHGGCGLHIP	*	** *
	*	*	*
	280	300	
pin.alpha	PNLPLSVPGAPPTPVQPLLLVPGAQPCCAALPGTMRSLRVRTTSDGGYSF		
hin.alpha	PNLSLPVPGAPPTPAQPYSLLPGAQPCCAALPGTMRPLHVRTTSDGGYSF	*	** *
	*	*	*
	320	334	
pin.alpha	KYETVPNLLTQHCACI		
hin.alpha	KYETVPNLLTQHCACI		

Fig. 8A.

1 TGCTCCCTGACAGCCACAACCTACAGCACTGACTGCATTTCAGAGAGGAACCTGCAAAACAACTTTCACAGAAAACCTTTTGTTCTGTTCAGAGAATTT

101 TGCTGAAGAGGAGAGGAAAAAACCCAAAAAATAAAAAATCCACACACACAAAAACCTGCGCTGAGGGGGGAGGAAAAAGCAGGGGCGCT

201 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG -28
Met Pro Leu Leu Trp Leu Arg Gly Phe Leu Leu Ala Ser Cys Trp
ATG CCC TTG CTT TGG CTG AGA GGA TTT CTG TTG GCA AGT TGC TGG

301 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG -1 +1
Ile Ile Val Arg Ser Ser Pro Thr Pro Gly Ser Glu Gly His Ser Ala Ala Pro Asp [] Pro Ser [] Ala Leu
282 ATT ATA GTG AGG AGT TCC CCC ACC CCA GGA TCC GAG GGG CAC AGC GCG GCC CCC GAC [] CCG TCC [] GCG CTG

401 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG -10 20
Ala Ala Leu Pro Lys Asp Val Pro Asn Ser Gln Pro Glu Met Val Glu Ala Val Lys Lys His Ile Leu Asn Met
357 GCC GCC CTC CCA AAG GAT GTA CCC AAC TCT CAG CAG ATG GTG GAG GCC GTC AAG AAG CAC ATT TTA AAC ATG

501 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG 40 50 60
Leu His Leu Lys Lys Arg Pro Asp Val Thr Gln Pro Val Pro Lys Ala Ala Leu Leu Asn Ala Ile Arg Lys Leu
432 CTG CAC TTG AAG AAG AGA CCC GAT GTC ACC CAG CCG GTA CCC AAG GCG GCG CTT CTG AAC GCG ATC AGA AAG CTT

601 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG 70 80
His Val Gly Lys Val Gly Glu Asn Gly Tyr Val Glu Ile Glu Asp Asp Ile Gly Arg Arg Ala Glu Met Asn Glu
507 CAT GTG GGC AAA GTC GGG GAG AAC GGG TAT GTG GAG ATA GAG GAT GAC ATT GGA AGG AGG GCA GAA ATG AAT GAA

701 TTTAAAAAGGCAATCACAACTTTTGCTGCCAGG 90 100
Leu Met Glu Gln Thr Ser Ser Glu Ile Ile Thr Phe Ala Glu Ser Gly Thr Ala Arg Lys Thr Leu His Phe Glu Ile
582 CTT ATG GAG CAG ACC TCG GAG ATC ATC ACG TTT GCC GAG TCA GGA ACA GCC AGG AAG ACG CTG CAC TTC GAG ATT

Fig.8B.

	120		130		140	150	160
Ser Lys Glu Gly Ser Asp Leu Ser Val Val Glu Arg Ala Glu Val Trp Leu Phe Leu Lys Val Pro Lys Ala Asn							ZZZ
657 TCC AAG GAA GGC AGT GAC CTG TCA GTG GTG GAG CGT GCA GAA GTC TGC CTC TTC CTA AAA GTC CCC AAG GCC AAC							
ZZZZZZ	140						
Arg Thr Arg Thr Lys Val Thr Ile Arg Leu Phe Gln Gln Lys His Pro Gln Gly Ser Leu Asp Thr Gly Glu							
732 AGG ACC AGG ACC AAA GTC ACC ATC CGC CTC TTC CAG CAG CAG AAG CAC CCG CAG GGC AGC TTG GAC ACA GGG GAA							
	170		180				
Glu Ala Glu Glu Val Gly Leu Lys Gly Glu Arg Ser Glu Leu Leu Ser Glu Lys Val Val Asp Ala Arg Lys							
807 GAG GCC GAG GAA GTG GGC TTA AAG GGG GAG AGG AGT GAA CTG TTG CTC TCT GAA AAA GTA GTA GAC GCT CGG AAG							
	190		200				210
Ser Thr Trp His Val Phe Pro Val Ser Ser Ser Ile Gln Arg Leu Leu Asp Gln Gly Lys Ser Ser Leu Asp Val							
882 AGC ACC TGG CAT GTC TTC CCT GTC TCC AGC AGC ATC CAG CGG TTG CTG GAC CAG GGC AAG AGC TCC CTG GAC GTT							
	220		230				
Arg Ile Ala GAA Glu Gln GAA Gln Gln Ser Glu Ser Gly Ala Ser Leu Val Leu Leu Gly Lys Lys Lys Lys Glu Glu							
957 CGG ATT GCC GAA GAG CAG GAA CAG GAG AGT GGC GCC AGC TTG GTT CTC CTG GGC AAG AAG AAG AAA GAA GAG							
	240		250				260
Glu Gly Glu Gly Lys Lys Lys Gly Gly Gly Glu Gly Ala Gly Ala Asp Glu Glu Lys Lys Glu Gln Ser His Arg							
1032 GAG GGG GAA GGG AAA AAG AAG GGC GGA GGT GAA GGT GGA GCA GCA GAT GAG GAA AAG GAG CAG TCG CAC AGA							

Fig.8C.

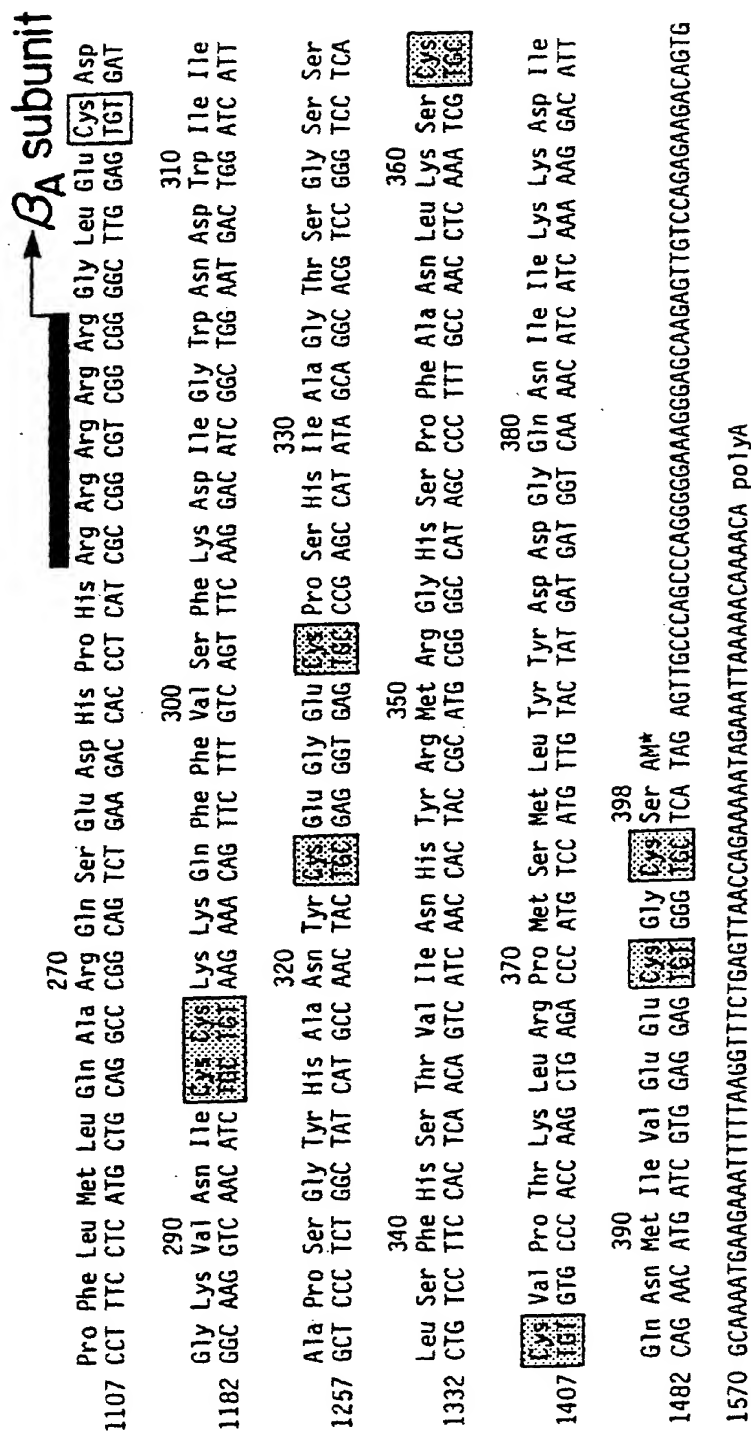


Fig. 9A.

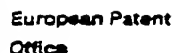
1 CC
 7 Cys Thr Ser Cys Gly Gly Phe Arg Arg Pro Glu Glu Leu Gly Arg Val Asp Gly Asp Phe Leu Glu Ala Val
 10 Cys Thr Ser Cys Gly GGC TTC CGG CGG CCA GAG GAG CTC GGC CGA GTG GAC GGC GAC TTC CTG GAG GCG GTG
 20
 30
 75 AAG CGG CAC CAC ATC TTG AGC CGC CTG CAG ATG CGG GGC Arg Gly Arg Pro Asn Ile Thr His Ala Val Pro Lys Ala Ala Met
 40
 50
 Val Thr Ala Leu Arg Lys Leu His Ala Gly Lys Val Arg Glu Asp Gly Arg Val Glu Ile Pro His Leu Asp Gly
 60
 70
 150 GTC ACG GCC CTG CGC AAG CTG CAC GCG GGC AAG GTG CCG GAG GAG GGC GGC GTG GAG ATC CCG CAC CTC GAC GGC
 80
 His Ala Ser Pro Gly Ala Asp Gly Glu Glu Arg Val Ser Glu Ile Ile Ser Phe Ala Glu Thr Asp Gly Leu Ala
 90
 100
 225 CAC GCC AGC CCG GGC GCC GAC GGC CAG GAG CGC GTT TCC GAA ATC ATC AGC TTC GCC GAG ACA GAT GGC CTC GCC
 110
 120
 300 TCC TCC CGG GTC CGC CTA TAC TTC ATC TTC AAC GGC AAC CAG AAC CTG TTT GTG GTC CAG GCC AGC CTG
 130
 Trp Leu Tyr Leu Lys Leu Leu Pro Tyr Val Leu Glu Lys Gly Ser Arg Arg Lys Val Arg Val Lys Val Tyr Phe
 140
 150
 375 TGG CTT TAC CTG AAA CTC CTG CCC TAC GTC CTG GAG AAG GGC AGC CGG AAG GTG CGG GTC AAA GTG TAC TTC
 160
 170
 450 Gln Glu Gln Gly His Gly Asp Arg Trp Asn Met Val Glu Lys Arg Val Asp Leu Lys Arg Ser Gly Trp His Thr
 180
 CAG GAG CAG GGC CAC GGT GAC AGG TGG AAC ATG GTG GAG AAG AGG GTG GAC CTC AAG CGC AGC GGC TGG CAT ACC

Fig.9B.

190 Phe Pro Leu Thr Glu Ala Ile Gln Ala Leu Phe Glu Arg Gly Glu Arg Arg Leu Asn Leu Asp Val Gln Cys Asp
 525 TTC CCA CTC ACG GAG GCC ATC CAG GCC TTG TTT GAG CGG GGC GAG CGG CGA CTC AAC CTA GAC GTG CAG TGG GAC
 200
 210 Ser Cys Gln Glu Leu Ala Val Val Pro Val Phe Val Asp Pro Gly Glu Glu Ser His Arg Pro Phe Val Val Val
 600 AGC TGG CAG GAG CTG GCC GTG GTG CCG GTG TTC GTG GAC CCA GGC GAA GAG TCG CAC CGG CCC TTT GTG GTG GTG
 220
 230 Gln Ala Arg Leu Gly Asp Ser Arg His Arg Ile Arg Lys Arg Gly Leu Glu Cys Asp Gly Arg Thr Asn Leu Cys
 675 CAG GCT CGG CTG GGC GAC AGC AGG CAC CGC ATT CGC AAG CGA GGC CTG GAG TGG GAT GGC CGG ACC AAC CTC TGG
 240
 250 Arg Gln Gln Phe Phe Ile Asp Phe Arg Leu Ile Gly Trp Asn Asp Trp Ile Ile Ala Pro Thr Gly Tyr Tyr
 750 TGG AGG CAA CAG TTC TTC ATT GAC TTC CGC CTC ATC GGC TGG AAC GAC TGG ATC ATA GCA CCC ACC GGC TAC TAC
 260
 270
 280
 290 Gly Asn Tyr Cys Glu Gly Ser Cys Pro Ala Tyr Leu Ala Gly Val Pro Gly Ser Ala Ser Ser Phe His Thr Ala
 825 GGG AAC TAC TGG GAG GGC AGC TGG CCA GCC TAC CTG GCA GGC GTC CCC GGC TCT GCC TCC TTC CAC ACG GCT
 300
 310 Val Val Asn Gln Tyr Arg Met Arg Gly Leu Asn Pro Gly Thr Val Asn Ser Cys Cys Ile Pro Thr Lys Leu Ser
 900 GTG GTG AAC CAG TAC CGC ATG CCG GGT CTG AAC CCC GGC ACG GTG AAC TCC TGG TGG ATT CCC ACC AAG CTG AGC
 320
 330
 340 Thr Met Ser Met Leu Tyr Phe Asp Asp Glu Tyr Asn Ile Val Lys Arg Asp Val Pro Asn Met Ile Val Glu Glu
 975 ACC ATG TCC ATG CTG TAC TTC GAT GAT GAG TAC AAC ATC GTC AAG CCG GAC GTG CCC AAC ATG ATT GTG GAG GAG
 350

Fig. 9C.

[illegible]



Appendix B

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 86307586.7
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D,P, X	WO - A1 - 86/00 078 (MONASH UNIVERSITY et al.) * Claims 1-6 *	1,12, 16,29, 31,36, 37	C 12 N 15/00 C 07 K 13/00 C 12 P 21/02
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 83, no. 16, August 1986, Baltimore, USA K.E.MAYO et al. "Inhibin A-subunit cDNAs from porcine ovary and human placenta" pages 5849-5853 * Totality *	1,12, 16	C 12 N 5/00 A 61 K 37/02 A 61 K 39/395
P,X	NATURE, vol. 318, 19/26 December 1985, New York, London A.J.MASON et al. "Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor- β " pages 659-663 * Totality *	1,13- 16,29- 33	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 N C 07 K C 12 P A 61 K
D,P, X	NATURE, vol. 321, 19 June 1986, New York, London W.VALE et al. "Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid" pages 776-779 * Totality *	1,12- 16,29- 33	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 16-02-1987	Examiner WOLF
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date O : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			



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EUROPEAN SEARCH REPORT

Application number

-2-

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 86307586.7
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claims	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D,P, X	NATURE, vol. 321, 19 June 1986, New York, London N.LING et al. "Pituitary FSH is re- leased by a heterodimer of the β - subunits from the two forms of inhibin" pages 779-782 * Totality * --	1,12, 13,15, 29-33	
D,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 82, no. 12, June 1985, Baltimore, USA C.H.LI et al. "Human seminal & inhibins; Isolation, characterization, and structure" pages 4041-4044 * Totality * --	12	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
D,X	FEBS LETTERS, vol. 175, no. 2, October 1984, Amsterdam N.G.SEIDAH et al. "Complete amino acid sequence of human seminal plasma β -inhibin" pages 349-355 * Totality * --	12	
E,X	WO - A1 - 86/06 076 (BIOTECHNOLOGY AUSTRALIA PTY.LTD. et al.) * Claims 1,6,18,24,30,37,39-41, 62 * ----	1,12- 28,37	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 16-02-1987	Examiner WOLF
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			